HUMAN PREPROTACHYKININ GENE PROMOTER

This application is a continuation of U.S. Application Number 09/747,429, filed December 23, 2000, now pending, which claims the benefit of U.S. Provisional Application No. 60/171,970, filed December 23, 1999. Both of these applications are

5   incorporated by reference herein in their entirety.

FIELD OF THE INVENTION

10   This invention relates to the fields of molecular biology, drug discovery and neoplastic transformation. More specifically, nucleic acid molecules encoding a preprotachykinin (*PPT*) gene promoter or 3' mRNA operably linked to a nucleic acid encoding a reporter molecule are provided for use in methods for identifying beneficial therapeutic reagents which influence expression levels and biochemical

15   functions of this protein. The compositions of the invention may be used to advantage in the discovery of therapeutic agents for the treatment of cancer and other hematologic disorders.

BACKGROUND OF THE INVENTION

The immune-hematopoietic-neural axis encompasses the neuroendocrine

20   system. These two systems cooperate via biochemical cross-talk. A particular cytokine or neuropeptide can be produced in cells of both neural and peripheral tissue, the latter including lymphoid organs and bone marrow, among others. Soluble factors mediate this bidirectional communication between the nervous and immune/hematopoietic systems. Due to this crosswalk, changes in one system often

25   influence functional changes in the other. In fact, studies show that the onset of hematopoiesis is correlated with complete innervation of the bone matter.

Stress, either physical, chemical or psychological, induces soluble brain derived factors and has been implicated in altered immune functions. Stress has also been associated with the incidence, relapse and prognosis of cancer. Specifically,

30   stress-induced neurohormones have been implicated in the development of breast

cancer. Biological homeostasis is achieved by the complex interacting network of all these soluble factors. The interactions of neurohormones and neurotransmitters are not mutually exclusive of the interactions exerted by cytokines and neurotrophic factors. Dysregulated and inappropriate expression of factors, such as

5   neuroendocrine-derived peptides, disrupts homeostasis. Ultimately, tumors may develop as a result of disrupted homeostasis. An example of this type of dysregulation is the constitutive expression of neurokinins observed in some tumors.

The Preprotachykinin-I (*PPT-I*) gene encodes a family of peptides that interact with a network of soluble factors in neural and non-neural tissues to exert

10   biological pleiotropism, such as neurotransmission, immune modulation and hematopoiesis (Rameshwar, P. 1997, *Clin. Immunol. Immunopath*, 85:129; Maggi, C.A. 1996, *Pharmacol. Res.* 33:161; Merril, J.E. *et al.* 1995, *FASEB J.*, 9:611). The role of *PPT-I* in hematopoiesis and angiogenesis, and its over expression in breast and other cancers that metastasize to the bone marrow, suggests that *PPT-I* has a central

15   role in bone marrow metastasis (Singh,D. *et al.* 2000, *Proc. Nat'l. Acad. Sci U.S.A.* 97:388; Hennig, I.M. *et al.* 1995, *Int. J. Cancer* 61:786; Jones, D.A. *et al.* 1997, *Peptides* 18:1073). The evolutionarily conserved sequence of PPT-I peptides underscores the importance of the pleiotropism that these peptides demonstrate in interactive biological functions (Moore, T.C. *et al.* 1990 *Immunopharmacol* 20:207).

20   The ability to manipulate and understand the regulation of *PPT-I* may help to facilitate modulation of the molecular mechanisms mediated by this gene that underlie organ specific functions and provide new insights into human pathology such as tumorgenesis, hematological diseases, nerve damage and other brain-associated functions and/or behavior.

25   Neurokinins are a family of neurotransmitters derived from expression of the preprotachykinin-I (*PPT-I*) gene. This gene is also expressed in a variety of non-neuronal cells. The *PPT1* gene is alternatively spliced into one of four transcripts, α, β, γ, and δ *PPT-I*. Each transcript can produce substance P (SP). The γ and δ *PPT-I* transcripts produce neurokinin-A (NK-A). Various N-terminally extended forms of

30   NK- are also expressed from the *PPT-I* gene.

Regulated expression of SP and NKA and other biological mediators act in concert to maintain homeostasis. Overproduction of SP and NKA can contribute to

neoangiogenesis, facilitating the growth of metastatic tumors. While SP exerts angiogenic properties directly, NK-A has the potential to exert indirect angiogenic function through TGF-β production. The elucidation of this relationship is desirable for developing therapeutic agents for the treatment of cancer. In particular, agents

5   which regulate the expression of *PPT-I* may be efficacious in the treatment of cancers and other hematopoietic disorders.

In the bone marrow, the two major PPT-I peptides, through their natural neurokinin receptors, NK-1 and NK-2, exert opposing influences, inhibitory and stimulatory, on hematopoiesis at the level of the mature and immature progenitors

10   (Rameshwar, P. *et al.* 1997, *Leuk. Lymphoma* 28:1). Therefore, hematopoietic stimulation by one of the major PPT-I peptides may be clinically important in hematologic deficiencies such as in the development of neutropenia and also other inflammatory responses (Cao, T. *et al.* 2000, *J. Immunol* 164:5424). The inhibitory effect could be important in protection of the lymphohematopoietic stem cells in the

15   bone marrow, where maintaining cell quiescence is often important. Furthermore, PPT-I is involved in the cellular and molecular connection among the immune, neuroendocrine and hematopoietic systems (Rameshwar, P. 1997, *supra.*). Thus, the regulation of PPT-I has relevance to bone marrow-associated biology, including the rapidly evolving fields of transplantation and gene therapy and also, inflammatory

20   processes.

Bone marrow fibrosis is a pathological secondary reaction which occurs in certain myeloproliferative disorders. Whereas the mechanisms that lead to bone marrow fibrosis are poorly understood, it has been hypothesized that soluble factors such as platelet derived growth factor (PDGF), transforming growth factor β (TGF-β)

25   and epidermal growth factor (EGF) act to induce deposits of extracellular matrix proteins in the bone marrow. TGFβ stimulates synthesis of collagen and fibronectin in fibroblasts and also augments the proliferation of human bone marrow fibroblasts by EGF and PDGF. The NK-1 receptor shares homology with regions of fibronectin. Preliminary studies show that substance P (SP), the preferred ligand for NK-1, binds

30   to fibronectin in the sera of patients with various categories of bone marrow fibrosis. SP is a fibrogenic factor and a stimulator of macrophages. As fibroblasts and macrophages are implicated in the pathophysiology of bone marrow fibrosis, SP is

transported to the bone marrow and other organs via binding to fibronectin. Accordingly, *PPT-I* (the gene from which SP is derived) provides a suitable target for treatment of patients with bone marrow fibrosis. Since bone marrow fibrosis is secondary to myeloproliferative disorders, *PPT-I* genes, their encoded peptides and

5   their receptors, and/or pathways associated with the products of these genes can also serve as targets to identify beneficial therapeutic reagents for treating patients with such disorders.

SUMMARY OF THE INVENTION

10   In one embodiment of the invention, an isolated polynucleotide comprising a *PPT–I* promoter region is provided. In one embodiment, this region occupies a segment of a human *PPT-I* gene between about 722 bases upstream from a transcription initiation site of the gene, and encompassing the transcription initiation site, as shown in Figure 2. Preferably, the *PPT-I* promoter is isolated from a gene

15   whose coding region hybridizes under moderate conditions with a coding region of a *PPT-I* gene, such as that shown in SEQ ID NO:15. Most preferably, this promoter region comprises SEQ ID NO:1. The *PPT-I* promoter sequence of the invention contains a plurality of CRE regulatory sequence elements, and other transcription factor responsive elements. Expression vectors comprising the *PPT -I* promoter

20   sequence operably linked to a heterologous nucleic acid molecule are also within the scope of the present invention. In a preferred embodiment of the invention, the heterologous nucleic acid molecule encodes a reporter gene. Reporter genes suitable for this purpose include, without limitation, β-galactosidase, chloramphenicol acetyltransferase, luciferase, secreted alkaline phosphatase and green fluorescent

25   protein.

In another embodiment of the present invention, an isolated polynucleotide comprising a *PPT-I* gene 3' end mRNA is provided. Preferably, the *PPT-I* 3' region is isolated from a gene whose coding region hybridizes under moderate conditions with a coding region of a *PPT-I* gene, such as that shown in SEQ ID NO:15. In a highly

30   preferred embodiment, the sequence of this polynucleotide comprises SEQ ID NO:4.

The present invention also features an isolated polynucleotide comprising a the upstream region of exon 1 of a gene encoding the neurokinin receptor, NK-2.

Preferably, this promoter is isolated from a gene having a coding region defined by the exons set forth in SEQ ID NO:2. More preferably, this upstream region is that shown in SEQ ID NO:2. The upstream *NK-2* sequence of the invention contains a plurality of CRE regulatory sequence elements, and other transcription factor

5   responsive elements. Expression vectors comprising the *NK-2* upstream sequence operably linked to a heterologous nucleic acid molecule are also within the scope of the present invention. In a preferred embodiment of the invention, the heterologous nucleic acid molecule encodes a reporter gene. Reporter genes suitable for this purpose include, without limitation, β-galactodosidase, chloramphenicol

10   acetyltransferase, luciferase, secreted alkaline phosphatase and green fluorescent protein.

Also included in the present invention is an isolated polynucleotide comprising a promoter and gene for the SP receptor (SP-R). Preferably, this promoter is isolated from a gene having a coding region defined by the exons as set

15   forth in SEQ ID NO:3. More preferably, this upstream region is that shown in SEQ ID NO:3. The upstream *SP-R* sequence of the invention contains a plurality of CRE regulatory sequence elements, and other transcription factor responsive elements. Expression vectors comprising the *SP-R* promoter operably linked to a heterologous nucleic acid molecule are also within the scope of the present invention. In a

20   preferred embodiment of the invention, the heterologous nucleic acid molecule encodes a reporter gene. Reporter genes suitable for this purpose include, without limitation, β-galactosidase, chloramphenicol acetyltransferase, luciferase, secreted alkaline phosphatase and green fluorescent protein.

In yet further aspect of the invention, an isolated host cell transformed with

25   the expression vectors described above is provided. Host cells contemplated for use in this aspect of the invention include procaryotic, eucaryotic, fungal, plant, mammalian and insect cells.

In a further aspect of the invention a process for producing a host cell containing a heterologous gene operably linked to the above mentioned sequences is

30   provided. The process comprises i) transfecting a cell with an expression vector comprising a heterologous gene operably linked to the sequence; ii) simultaneously transfecting the cell with a selectable marker gene which confers resistance to a

selection agent; and iii) selecting and isolating transformed host cells on the basis of resistance to the selection agent, the host cell also containing a heterologous gene operably linked to the inventive sequence. In this embodiment of the invention, the selectable marker gene may or may not be on the same expression construct as the

5   inventive sequence/reporter gene construct.

Methods utilizing the expression constructs and host cells containing the same for identifying agents which affect the promoter activity of the *PPT-1*, or the *NK-2* gene are also provided in the present invention. Similarly, methods utilizing the expression constructs and host cells containing the same for identifying agents which

10   affect activity of the *SP* receptor (SP-R) gene are provided in the present invention. Transformed host cells are contacted with an agent which inhibits or stimulates promoter activity. Influence of the test agent on promoter function is determined based on levels of expression of the reporter gene relative to the appropriate negative controls.

15   Also included in the present invention are methods for screening the *PPT-1* promoter, *NK-2* upstream sequence or *SP-R* gene for mutations associated with pathological conditions.

Preferably, the polynucleotides of the invention comprise a sequence selected from the following group: SEQ ID NO:1; an allelic variant of SEQ ID NO:1; a

20   sequence hybridizing with SEQ ID NO:1 or its complement under moderate hybridization and washing conditions; SEQ ID NO:2; an allelic variant of SEQ ID NO:2; a sequence hybridizing with SEQ ID NO:2 or its complement under moderate hybridization and washing conditions; SEQ ID NO:3; an allelic variant of SEQ ID NO:3; a sequence hybridizing with SEQ ID NO:3 or its complement under moderate

25   hybridization and washing conditions; SEQ ID NO:4; and an allelic variant of SEQ ID NO:4; a sequence hybridizing with SEQ ID NO:4 or its complement under moderate hybridization and washing conditions.

Another aspect of the invention features a recombinant DNA molecule comprising a vector having an insert that includes part or all of a *PPT-1* promoter or 3'

30   sequence, *NK-2* upstream sequence, or *SP-R* promoter and/or gene and cells transformed with any of the above-mentioned recombinant DNA molecule.

Preferably, the cells are human cells. Most preferably, the cells are from breast tissue cells, breast cancer cells, fibroblast cells, or epithelial cells.

The invention also features an isolated polypeptide produced by expression of the polynucleotides described above. Antibodies immunologically specific for the protein, or one or more epitopes thereof, are also provided.

In another aspect, the invention relates to methods for using such polypeptides and polynucleotides, including the treatment of breast cancer, bone marrow metastasis, pain, asthma, arthritis, aggressive behavior, and depression, where one or more of PPT-I peptides, NK-2 or SP-R is known to play a role. In still another aspect, the invention relates to methods to identify agonists and antagonists using the materials provided by the invention, and treating diseases or conditions associated with PPT-I, NK-2 or SP-R imbalance with the identified compounds.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the *PPT* gene showing the alternative splicing of the PPT mRNA transcript. Also depicted is the promoter region of the *PPT* gene.

Figure 2 depicts the sequence of the longest fragment cloned upstream of Exon 2, *PPT-I* (SEQ ID NO:14, which is a variant of SEQ ID NO:1). Nucleotides are numbered relative to exon 1. The TATA box is in bold. There are 722 nt upstream of Exon 1. Underlined sequences show consensus sequences for CRE, the 5' ends of Exon 1 (+1 /+89) and Intron 1 (+90/+498). Consensus sequences for NF-κB, shown by double underline, are found towards the end of the 5' region and within Exon 1. GenBank Accession Number AF252261.

Figure 3 shows Luciferase activities by *PPT-I-p1.2* fragments in bone marrow stroma. Different fragments of *PPT-I-p1.2* were ligated in pGL3, upstream of the luciferase gene and then transfected in bone marrow stroma. The results are the mean (±SD) of 7 experiments; each performed with a different bone marrow donor.

Figure 4. Fig. 4A is a representative gel shift of four different experiments showed that CRE and CRE-like can bind ICERIIγ using wild type or mutant oligonucleotide probes. Lanes 1: wild type; 2: mutant; 3 and 4: 200 and 50 ng mutant cold competitor respectively; 5, 6 and 7: 200, 100 and 50 ng wild type cold

competitor respectively. (▷) protein-DNA interactions. Figs. 4B, 4C and 4D are representative western blots of three different experiments were determined for CRE-binding proteins in bone marrow stroma with an antibody that reacts with CREM (B: top arrow) and ICERIIγ (B: lower arrow), anti CREB (C) or anti-phospho CRE-

5   activators (D: top lanes, CREM and CREB; bottom lanes: ATF-1). Extreme left lanes: molecular weight marker. Lanes 1: unstimulated stroma; 2: FK-2h; 4: FK-5h.

Figure 5 shows representative data of six different experiments that were performed with bone marrow stroma, transiently transfected with pGL3 basic-Upstream/N0 that contained wild type or mutant CRE and/or mutant CRE-like (Table

10   1). Cells were co-transfected with 200 ng/µl of pSV that expressed: 1. PKA and CREMτ, 2. PKA or, 3. PKA, CREMτ and ICERIIγ. Fig. 5A: Western blots with rabbit antibodies verified that the transfected CREMτ and ICERIIγ were expressed in the co-transfected cells, representative figure shows; Lanes 1: no transfection, 2 and 5: CREMτ, 3 and 6: ICERIIγ and 4: salmon sperm DNA. Fig. 5B: Reporter activity

15   was determined 48 h after transfection in cell lysates. * $p<0.05$ vs. Upstream/N0 or PKA, CREMτ, ICERIIγ.

Figure 6 shows the effects of CRE and CRE-like in the induction of Upstream/N0. Bone marrow stroma was transfected with pGL3-Upstream/N0 containing wild type CRE, mutant CRE, mutant CRE-like or double mutant (CRE and

20   CRE-like) and then stimulated with optimal SCF or IL-1α (Fig. 6A). In parallel studies, stroma was co-transfected with Upstream/N0 containing wild type CRE and CRE-like and, CREMτ or ICERIIγ. Stroma was stimulated 24 h after transfection with optimal SCF or IL-1α, Table 2 (Fig. 6B). After 10 h luciferase activity was determined in cell lysates. Stimulation time was deduced from time-course studies

25   ranging from 2 to 48 h. Fig. 6A: ** $p<0.05$ vs. unstimulated stroma, * $p<0.05$ vs. wild type CRE. Fig. 8B: * $p<0.05$ vs. CREMτ, ** $p<0.05$ vs. Upstream/N0 or Upstream/N0, CREMτ. Each experimental point is the mean (±SD) of duplicate transfections with 7 bone marrow donors.

Figure 7 shows the effects of NK-1R antagonist on the activation of *PPT-I*

30   promoter by SCF. Fig. 7A: bone marrow stromal cells, transfected with pGL3-Upstream/N0 were stimulated with 8 ng/ml SCF or 2.5 ng/ml IL-1α for 36 h. During

the middle of the incubation period, culture media with cytokines were replaced. After this, cells were washed and then incubated within 0.1 nM CP-99,994. After 8 h, luciferase activity was determined in cell lysates as described for Figure 6. Fig. 7B: Model to explain indirect stimulation of *PPT-I* by a representative cytokine. SCF-

5   receptor interaction leads to induction of *PPT-I* and *NK-1*. The peptides derived from PPT-I transcription are released and interact with the G-protein coupled NK-1 on the cell membrane. PPT-I-peptide-NK-1 interaction results in activation of cAMP pathway, which activates putative kinase(s), thus activation of the two CRE regions within PPT-I promoter. *$p<0.01$ vs. unstimulated or SCF+CP-99,994. © cAMP

10 pathway; ® Receptor.

Figure 8 depicts fibroblasts (CRL 1502) or 5x passaged primary bone marrow adherent cells and normal mammary epithelial cells, MCF-10 and MCF-12A were transfected with pGL3-*PPT-I*-p1.2 or pGL3-Upstream/N0. The techniques for luciferase activity and normalization with β-gal are described in Figure 3. The results

15 are represented as the ratio of luciferase induction in cells transfected with *PPT-I*-p1.2 over Upstream/N0. *$p<0.05$ vs. fibroblast or SY5Y, n=7, ±SD.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are provided to facilitate an understanding of the present invention.

20 "*PPT-I* gene" or "*PPT-I*" refers to a polynucleotide as defined above in accordance with the present invention, which encodes PPT-I peptides.

"PPT-I" or "PPT-I peptides" refer generally to peptides or polypeptides encoded by *PPT-I*.

"PPT-I activity or PPT-I polypeptide activity" or "biological activity of

25 the PPT-I or PPT-I polypeptide" refers to the metabolic or physiologic function of said PPT-I including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said PPT-I.

"*NK-2*" or "*NK-2* gene" refers to a polynucleotide as defined above in

30 accordance with the present invention, which encodes an NK-2 polypeptide.

"NK-2" refers generally to a polypeptide encoded by *NK-2*, which is described in detail herein above and throughout the specification. NK-2 is a neurokinin receptor, and is also sometimes referred to herein as "NK-2R", and the terms are meant to be used interchangeably.

5   "NK-2 activity or NK-2 polypeptide activity" or "biological activity of the NK-2 or NK-2 polypeptide" refers to the metabolic or physiologic function of said NK-2 including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said NK-2.

10   NK-1 is also a neurokinin receptor and its regulation in various cell types is discussed in detail herein. NK-1 is also sometimes referred to herein as "NK-1R", and the terms are meant to be used interchangeably.

"*SP-R* gene" refers to a polynucleotide which encodes an SP receptor polypeptide.

15   "SP-R" or "Substance P receptor" refers generally to a polypeptide encoded by *SP-R*, which is described in detail herein above and throughout the specification.

"SP-R activity or SP-R polypeptide activity" or "biological activity of the SP-R or SP-R polypeptide" refers to the metabolic or physiologic function of said SP-R including similar activities or improved activities or these activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of said SP.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library. With respect to antibodies, the term, "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

30   "Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a

polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

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"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or,

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more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated

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bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as

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oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as

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proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research

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literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying

degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslation natural processes or

5   may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of

10   covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

15   See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for

20   protein modifications and nonprotein cofactors", *Meth Enzymol* (1990) 182:626-646 and Rattan *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci* (1992) 663:48-62.

      "Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains

25   essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded

30   by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the

variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of

5   a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

The term "substantially the same" refers to nucleic acid or amino acid

10 sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate

15 codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

20 The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "identity" or "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program.

25 "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor

30 (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

"Identity" and "similarity" can be readily calculated by known methods. Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. In preferred methodologies, the BLAST programs (NCBI) and parameters used therein are employed, and the DNAstar system (Madison, WI) is used to align sequence fragments of genomic DNA sequences. However, equivalent alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

With respect to single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

With respect to oligonucleotides, but not limited thereto, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and

most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate to the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

A "coding sequence" or "coding region" refers to a nucleic acid

5   molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so

10   as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals,

15   terminators, and the like, that provide for the expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns.

20   Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels

25   detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to

30   which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, a heterologous region is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein. The term "DNA construct", as defined above, is also used to refer to a heterologous region, particularly one constructed for use in transformation of a cell.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter

cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

As used herein, the term "test subject" or "patient" shall include both

5   mammals and humans.

Neuropeptides have been observed in many tumors, including breast cancer. The preprotachykinin-I gene (*PPT-I*) encodes multiple neuropeptides that exert pleiotropic functions, including neurotransmission, immune/hematopoietic

10   modulation, angiogenesis and mitogenesis. *PPT-I* is constitutively expressed in some tumors. In accordance with the present invention, a role for PPT-I and its receptors, NK-1 and NK-2, in breast cancer has been determined, using quantitative RT-PCR, ELISA and *in situ* hybridization. In breast cancer cell lines and malignant breast biopsies, increased expression of *PPT-I, NK-1* and *NK-2* genes was observed. In

15   normal mammary epithelial cells and benign breast biopsies, NK-2 levels are elevated, while NK-1 and PPT- I peptides are not detected. Specific NK-1 and NK-2 antagonists inhibit breast cancer cell proliferation, suggesting autocrine and/or intercrine stimulation of breast cancer cells by PPT-I peptides. Unlike normal cells where NK-2 maintains cell quiescence, NK-2 mediates cell proliferation in breast

20   cancer cells.

Further, it was discovered in the present invention that cytosolic extracts from malignant breast cancer cells enhance *PPT-I* translation, whereas extracts from normal mammary epithelial cells fail to enhance translation. These enhancing effects of malignant cell extracts on *PPT-I* appear to be protein specific, as a similar increase

25   was observed for IL-6 translation, but no effect was detected for IL-1α and SCF translation. These findings further implicate a role for PPT-I peptides and their receptors in the development of breast cancer. Considering that PPT-I peptides are hematopoietic modulators, these results provide insight into the events leading to early integration of breast cancer cells in the bone marrow, a preferred site of

30   metastasis. The manipulation of molecular signaling transduced by PPT-I peptides and the mechanism that enhances translation of PPT-I mRNA enable development of innovative strategies for treating breast cancer and metastasis.

As described in greater detail herein, the inventors have cloned the genomic sequences upstream of the coding region of human *PPT-I*, and identified the area with promoter activity. Several modulators of PPT-I regulation activate the cAMP pathways. The inventors have discovered the consensus sequences for two cAMP

5   response elements (CRE) in the promoter region of *PPT-I*, which are involved in the regulation of *PPT-I* expression. Several transcription factors can bind as dimers to CRE: CREM (CRE modulator), CREB (CRE binding proteins) and activator transcription factor-1, ATF-1 (Molina, C.A. 1997. J. L. Tilly, et al.,Verlag, NY. p. 182; Sassone-Corsi, P. 1995., *Annu. Rev. Cell. Dev. Biol* 11:355). CRE-interacting

10   proteins are primarily constitutive, and their activation requires cAMP-dependent PKA (Molina, C.A. *supra*). A second internal promoter in the CREM gene can be induced by cAMP to produce a repressor, ICER (inducible cAMP early repressor), which is a negative regulator of cAMP-induced transcription (Molina,C.A. *supra*; Sassone-Corsi, P., *supra*). In accordance with the present invention, areas of

15   mutations in the promoter of *PPT-I* gene associated with breast cancer, breast cancer metastasis and certain hematopoietic disorders have been identified.

In organs where *PPT-I* is important to maintain steady-state functions, cytokines are important for regulating its expression (Rameshwar, P. 1997, *supra*). Therefore, the present invention utilizes representative cytokines to determine the role

20   of CRE and CRE-like sequences in cytokine-mediated *PPT-I* regulation. Cytokines are believed to activate the cAMP pathway through direct and/or indirect mechanisms (Masuda, E.S. *et al.* 1993. *The Immunologist* 1/6:198). Indirect stimulation could occur through the induction of other soluble factors that can stimulate the cells through autocrine and/or paracrine mechanisms. The inventors have shown that IL-

25   1α and stem cell factor (SCF) require the two CREs for optimal promoter activity. The use of a specific NK-1 antagonist demonstrates that SCF induces *PPT-I* through direct and/or indirect mechanisms. The involvement of NK-1 in indirect induction of *PPT-I* by SCF is explained in a two-step mechanism: Concomitant induction of *PPT-I* and *NK-1* followed by auto stimulation of the expressed, membrane bound NK-1

30   with the released peptides derived from PPT-I. Since *PPT-I* is implicated in several functions, we determined tissue-specific expression. Two relevant cell types, fibroblasts and epithelial cells, show cell-specific differences in reporter activity.

Also in accordance with the present invention, upstream regulatory sequences of the *NK-2* gene and a gene encoding the SP receptor have been cloned. The control of expression of these genes is involved in the regulation of cell proliferation and malignancy.

5   The NK-1 receptor shares homology with regions of fibronectin, an extracellular matrix protein that is increased in patients with bone marrow fibrosis. It is shown herein that substance P (SP), the preferred ligand for NK-1, binds to fibronectin in the sera of patients with various categories of bone marrow fibrosis. SP is a fibrogenic factor produced by expression of the *PPT-I* gene and a stimulator of

10   macrophages. As fibroblasts and macrophages are implicated in the pathophysiology of bone marrow fibrosis, it appears that SP is transported to the bone marrow and other organs via binding to fibronectin. Thus, in one embodiment of the present invention, use of *PPT-I* (the gene from which SP is derived) is utilized as a target for treatment of patients with bone marrow fibrosis. Since bone marrow fibrosis is

15   secondary to myeloproliferative disorders, PPT-I and its receptor, and/or pathways associated with the products of these genes provide biological targets for the development of therapeutic agents for use in patients with myeloproliferative disorders.

        As set forth in Example 5 below, cytosolic factors present in malignant cells

20   enhance translation of a subset of genes. In accordance with the present invention, the identity and specificity of these translation enhancing factors is determined. Initially, it is ascertained which portions of the 3' sequence of PPT-I mRNA are important for the observed enhanced translation by the cytosolic extracts. Computer analyses can also be performed to assess the secondary folding of the mRNA for each

25   PPT-I transcript. Different sequences can be incubated with cytosolic factors and then separated by gel shift. Specificity of competition can be verified with the antisense sequences and with sequences from other regions of PPT-I mRNA. Once the relevant sequences are identified, affinity columns can be prepared with the relevant nucleotides and the putative cytosolic factors isolated. The proteinaceous

30   character of the factors can be confirmed followed by partial amino acid sequencing. Based on this sequence information, degenerate DNA probes can be synthesized and used to screen cDNA libraries to get the full length factor(s) coding sequence(s). In

the event that interacting proteins are identified, the first isolate will be utilized to retrieve others using the yeast two hybrid system.

Thus, the present invention provides expression regulatory elements for several key proteins involved in cell proliferation. In addition, the discoveries made

5   in accordance with the invention targets have revealed novel targets for development of therapeutic agents for the treatment of various forms of cancer, including myeloproliferative disorders, as well as bone marrow fibrosis. The sections below set forth various embodiments for practicing the present invention. To the extent that specific methods and/or reagents are specified, this is done for illustration only, and is

10   not intended to limit the invention.

Polynucleotides

The polynucleotides of the present invention include isolated polynucleotides

15   comprising the *PPT-I, NK-2* and *SP-R* promoters and/or 3' untranslated region, and fragments, and polynucleotides closely related thereto. More specifically, the polynucleotides of the invention include a polynucleotide comprising the human nucleotide sequences contained in SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO:3 or SEQ ID NO:4, and polynucleotides having the particular sequence of SEQ ID NO:1, SEQ ID

20   NO:2, SEQ ID NO:3 or SEQ ID NO:4. The polynucleotides further include a polynucleotide comprising a nucleotide sequence that is at least 70% identical to that of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4, over its entire length. In this regard, polynucleotides with at least 70% are preferred, more preferably at least 80% identity, even more preferably at least 90% identity, yet more preferably at

25   least 95% identity, 97% are highly preferred and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred. Also included are a nucleotide sequence which has sufficient identity to a nucleotide sequence contained in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4 to hybridize under conditions useable for amplification or for use as a probe or marker. The invention

30   also provides polynucleotides which are complementary to such PPT-I, NK-2 and SP polynucleotides.

When the polynucleotides of the invention are used as regulatory elements for the recombinant production of PPT-I, NK-2 or SP-R polypeptide, or for a heterologous polypeptide, the polynucleotide may include the coding sequence for the mature polypeptide or a fragment thereof, by itself; the coding sequence for the mature

5   polypeptide or fragment in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. For example, a marker sequence which facilitates purification of the fused polypeptide can be encoded. The polynucleotide may also contain other non-coding 5' and 3' sequences, such as transcribed, non-translated

10   sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention. Such oligonucleotides are useful as

15   probes for detecting *PPT-I, NK-2* or *SP-R* genes. In one preferred embodiment, oligonucleotides for use as probes or primers are based on rationally-selected nucleic acid sequences chosen from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4.

PPT-I, NK and *SP-R* polynucleotides of the present invention may be prepared

20   by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art. The availability of nucleotide sequence information, such as the cDNA having SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, enables preparation of an isolated nucleic acid molecule of the

25   invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides should be synthesized in stages, due to the

30   size limitations inherent in various oligonucleotide synthetic methods. Thus, for example, a long double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced

may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire long double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned

5   and amplified in an appropriate vector.

PPT-I, NK-2 and SP-R genes (or portions thereof) and promoters also may be isolated from appropriate biological sources using methods known in the art. In the exemplary embodiment of the invention, PPT-I, NK-2 and SP-R may be isolated from genomic libraries of human. A preferred means for isolating PPT-I, NK-2 and SP-R

10   genes is PCR amplification using genomic templates and sequence-specific primers. Genomic libraries are commerically available, and can also be made by procedures well known in the art. In positions of degeneracy where more than one nucleic acid residue could be used to encode the appropriate amino acid residue, all the appropriate nucleic acid residues may be incorporated to create a mixed

15   oligonucleotide population, or a neutral base such as inosine may be used. The strategy of oligonucleotide design is well known in the art.

In accordance with the present invention, nucleic acids having the appropriate level sequence homology (i.e., 70% identity or greater) with part or all of SEQ ID NO:1 SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4 may be identified by using

20   hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 1.0% SDS, up to 50% formamide, 5x SSC (150mM NaCl, 15mM trisodium citrate), 0.05% sodium pyrophosphate (pH7.6), 5x Denhardt's solution, and 100 microgram/ml denatured, sheared salmon sperm DNA. Hybridization

25   is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes to 1 hour at 37°C in 2X SSC and 0.1% SDS; (4) 2 hours at 45-55 °C in 2X SSC and 0.1% SDS, changing the solution every 30 minutes.

30   One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified percent identity is set forth by (Sambrook et al., 1989, supra):

$$T_m = 81.5 \,°C + 16.6 Log\,[Na+] + 0.41(\%\,G+C) - 0.63\,(\%\,formamide) - 600/\#bp\;in\;duplex$$

As an illustration of the above formula, using [N+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57 °C. The

5   T_m of a DNA duplex decreases by 1 - 1.5 °C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42 °C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of

10   annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20 - 25 °C below the calculated T_m of the of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12 – 20 °C below the T_m of the hybrid. In regards to the nucleic acids

15   of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42 °C, and wash in 2X SSC and 0.5% SDS at 55 °C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm

20   DNA at 42 °C, and wash in 1X SSC and 0.5% SDS at 65 °C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, SX Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42 °C, and wash in 0.1X SSC and 0.5% SDS at 65 °C for 15 minutes.

Nucleic acid molecules of the invention include cDNA (where appropriate),

25   genomic DNA, RNA, and fragments thereof which may be single or double stranded. Thus, this invention provides oligonucleotides having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the DNA having SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4. Primers capable of specifically amplifying

30   the nucleic acids of the invention are described herein. As mentioned previously, such oligonucleotides are useful as probes and primers for detecting, isolating or amplifying *PPT, NK-2* or *SP-R* genes.

Antisense nucleic acid molecules may be targeted to translation initiation sites, promoter regions, 3' untranslated regions and/or splice sites to inhibit the expression of the *PPT-I*, *NK-2* or *SP-R* genes or production of the encoded polypeptides. Such antisense molecules are typically between 15 and 30 nucleotides in length and often

5   span the translational start site of mRNA molecules. Suitable *PPT-I* antisense molecules for practicing this aspect of the invention include:

EXON 2:   GTG GAC ACA AGA AAA AAG ACT GCC A (SEQ ID NO:5)

EXON 3:   GAA GAT GCT CAA AGG CGT CCG GCA G (SEQ ID NO:6)

EXON 7:   ATA ATT CTG CAT TGC ACT CCT TTC AT (SEQ ID NO:7).

10   Alternatively, antisense constructs may be generated which contain the entire *PPT-I*, *NK-2* or *SP-R* promoters (or 3' untranslated region of *PPT-I*), with coding sequences operatively linked thereto, encoding the alternatively spliced PPT α, β, γ or δ proteins, NK-2 or SP-R proteins, in reverse orientation. Such antisense constructs are easily prepared by one of ordinary skill in the art.

15   It will be appreciated by persons skilled in the art that variants (e.g., allelic variants) of *PPT-I*, *NK-2* or *SP-R* sequences exist in the human population, and must be taken into account when designing and/or utilizing oligonucleotides of the invention. Accordingly, it is within the scope of the present invention to encompass such variants, with respect to the nucleotide sequences disclosed herein or the

20   oligonucleotides targeted to specific locations on the respective genes or RNA transcripts. Accordingly, the term "natural allelic variants" is used herein to refer to various specific nucleotide sequences of the invention and variants thereof that would occur in a human population. Such variants would not demonstrate altered *PPT-I*, *NK-2* or *SP-R* regulatory activity. Additionally, the term "substantially

25   complementary" refers to oligonucleotide sequences that may not be perfectly matched to a target sequence, but such mismatches do not materially affect the ability of the oligonucleotide to hybridize with its target sequence under the conditions described.

Nucleic acids of the present invention may be maintained as DNA in any

30   convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell.

The polynucleotides may be used for a variety of purposes in accordance with the present invention. DNA, RNA, or fragments thereof may be used as probes to detect the presence of and/or expression of *PPT-I, NK-2* or *SP-R* genes. Methods in which *PPT-I, NK-2* or *SP-R* nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) in situ hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reaction (PCR).

The *PPT-I, NK-2* and *SP-R* nucleic acids may also be utilized as probes to identify related genes from other species. As is well known in the art, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology.

The *PPT-I, NK-2* and *SP-R* nucleic acids of the present invention can be used to identify and isolate other members of the growth regulatory pathway(s) in which PPT-I, NK-2 or SP-R is involved. A yeast two-hybrid system can be used to identify proteins that physically interact with the PPT-I, NK-2 or SP-R protein, as well as isolate their nucleic acids. In this system, the coding sequence of the protein of interest is operably linked to the coding sequence of half of an activator protein. This construct is used to transform a yeast cell library which has been transformed with DNA constructs that contain the coding sequence for the other half of the activator protein operably linked to a random coding sequence from the organism of interest. When the protein made by the random coding sequence from the library interacts with the protein of interest, the two halves of the activator protein are physically associated and form a functional unit that activates the reporter gene. In accordance with the present invention, all or part of the human *PPT-I, NK-2,* or *SP-R* promoter or 3' end and/or coding sequence may be operably linked to the coding sequence of the first half of the activator, and the library of random coding sequences may be constructed with cDNA from human and operably linked to the coding sequence of the second half of the activator protein. Several activator protein/reporter genes are customarily used in the yeast two hybrid system, the Gal4/LacZ system (see Clark et al., 1998 PNAS 95:5401-5406), among others.

Vectors, Host Cells, and Expression

The present invention also relates to vectors which comprise a polynucleotide or polynucleotides of the present invention, and host cells which are genetically engineered with vectors of the invention and to the production of gene products by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis et al., *BASIC METHODS IN MOLECULAR BIOLOGY* (1986) and Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

A number of cell lines are particularly suited for the present invention and may be purchased from American Type Culture Collection (ATCC), Rockville, MD. These highly preferred cell lines are ZR7530, infiltrating ductal carcinoma from ascites fluid; BT474, ductal carcinoma; T47D, ductal carcinoma from pleural effusion; MDAMB330, breast carcinoma from pleural effusion; 184B5, chemically transformed mammary epithelial; DU4475 breast carcinoma; BT 483, ductal carcinoma; MCF12A, nontransformed mammary epithelial cells; Hs578Bst, normal breast epithelial cells; CCL64, Mink Lung epithelial; L929, murine fibroblast; and

MDBK, bovine epithelial kidney cell. All cell lines were cultured according to ATCC instructions.

Undifferentiated neuroblastoma cells, SH-SY5Y obtained from the Department of Biochemistry, UMDNJ-New Jersey Medical School, Newark, NJ are 5 particularly preferred for certain aspects of the present invention. SH-SY5Y cells should be cultured in DMEM with high glucose (Life Technologies, Grand Island, NY) containing 10% FCS (Hyclone Laboratories, Logan, UT). Skin fibroblasts (CRL 1502) and normal mammary epithelial cells (MCF-10 and MCF-12A) may be purchased from American Type Culture Collection (Manassas, VA). Cells should be 10 cultured based on their instructions.

More particularly, the present invention also includes recombinant constructs comprising one the promoter or 3' regulatory sequences of the invention. The constructs comprise a vector, such as a plasmid or viral vector, into which is inserted a DNA construct comprising a coding sequence operably linked to one of the 15 regulatory sequences of the invention. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. A variety of vectors, both viral vectors and plasmid vectors are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpes viruses 20 including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have employed disabled murine retroviruses. The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); 25 Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host. In addition, a complete mammalian transcription unit and a selectable marker can be inserted into a prokaryotic plasmid. The resulting vector is then amplified in bacteria before being transfected into 30 cultured mammalian cells. Examples of vectors of this type include pTK2, pHyg and pRSVneo.

A great variety of expression systems can be used. Such systems include, among others, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as

5   baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to

10   maintain, propagate or express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL* (*supra*).

15

Detection Of PPT-I Encoding Nucleic Acids And Assay Methods For Identifying Mutations, Antagonists And Agonists Affecting PPT-I Gene Expression

In accordance with the present invention, *PPT-I* expression has been found to be upregulated in breast cancers. Mutations in the promoter region or the coding

20   sequence of *PPT-I* are associated with the malignant phenotype. On one aspect of the invention, the *PPT-I* promoter and coding sequence isolated from cancer cells are screened for mutations. Currently, the most direct method for mutational analysis is DNA sequencing, however it is also the most labor intensive and expensive. It is usually not practical to sequence all potentially relevant regions of every experimental

25   sample. Instead, some form of preliminary screening method is commonly used to identify and target for sequencing only those samples that contain mutations. Single stranded conformational polymorphism (SSCP) is a widely used screening method based on mobility differences between singlestranded wild type and mutant sequences on native polyacrylamide gels. Other methods are based on mobility differences in

30   wild type/mutant heteroduplexes (compared to control homoduplexes) on native gels (heteroduplex analysis) or denaturing gels (denaturing gradient gel electrophoresis). Sample preparation is relatively easy in these assays, and conditions for

electrophoresis required to generate the often subtle mobility differences that form the basis for identifying the targets that contain mutations are well known to those of skill in the art. Another parameter to be considered is the size of the target region being screened. In general, SSCP is used to screen target regions no longer than about 200-

5   300 bases.

Another type of screening technique currently in use is based on cleavage of unpaired bases in heteroduplexes formed between wild type probes hybridized to experimental targets containing point mutations. The cleavage products are also analyzed by gel electrophoresis, as subfragments generated by cleavage of the probe

10   at a mismatch generally differ significantly in size from full length, uncleaved probe and are easily detected with a standard gel system. Mismatch cleavage has been effected either chemically (osmium tetroxide, hydroxylamine) or with a less toxic, enzymatic alternative, using RNase A. The RNase A cleavage assay has also been used, although much less frequently, to screen for mutations in endogenous mRNA

15   targets for detecting mutations in DNA targets amplified by PCR. A mutation detection rate of over 50% was reported for the original RNase screening method.

Another method to detect mutations in DNA relies on DNA ligase which covalently joins two adjacent oligonucleotides which are hybridized on a complementary target nucleic acid. The mismatch must occur at the site of ligation.

20   As with other methods that rely on oligonucleotides, salt concentration and temperature at hybridization are crucial. Another consideration is the amount of enzyme added relative to the DNA concentration. In summary, exemplary approaches for detecting alterations in *PPT-I* nucleic acids or polypeptides/proteins include:

25   a) comparing the sequence of nucleic acid in the sample with the wild-type *PPT-I* nucleic acid sequence to determine whether the sample from the patient contains mutations; or

        b) determining the presence, in a sample from a patient, of the polypeptide encoded by the *PPT-I* gene and, if present, determining whether the polypeptide is

30   full length, and/or is mutated, and/or is expressed at the normal level; or

        c) using DNA restriction mapping to compare the restriction pattern produced when a restriction enzyme cuts a sample of nucleic acid from the patient with the

restriction pattern obtained from normal *PPT-I* gene or from known mutations thereof; or,

d) using a specific binding member capable of binding to a *PPT-I* nucleic acid sequence (either normal sequence or known mutated sequence), the specific binding member comprising nucleic acid hybridizable with the *PPT-I* sequence, or substances comprising an antibody domain with specificity for a native or mutated *PPT-I* nucleic acid sequence or the polypeptide encoded by it, the specific binding member being labeled so that binding of the specific binding member to its binding partner is detectable; or,

e) using PCR involving one or more primers based on normal or mutated *PPT-I* gene sequence to screen for normal or mutant *PPT-I* gene in a sample from a patient.

A "specific binding pair" comprises a specific binding member (sbm) and a binding partner (bp) which have a particular specificity for each other and which in normal conditions bind to each other in preference to other molecules. Examples of specific binding pairs are antigens and antibodies, ligands and receptors and complementary nucleotide sequences. The skilled person is aware of many other examples and they do not need to be listed here. Further, the term "specific binding pair" is also applicable where either or both of the specific binding member and the binding partner comprise a part of a large molecule. In embodiments in which the specific binding pair are nucleic acid sequences, they will be of a length to hybridize to each other under conditions of the assay, preferably greater than 10 nucleotides long, more preferably greater than 15 or 20 nucleotides long.

In most embodiments for screening for susceptibility alleles, the *PPT-I* nucleic acid in the sample will initially be amplified, e.g. using PCR, to increase the amount of the analyte as compared to other sequences present in the sample. This allows the target PPT sequences to be detected with a high degree of sensitivity if they are present in the sample. This initial step may be avoided by using highly sensitive array techniques that are becoming increasingly important in the art.

The identification of the *PPT-I* gene's association with cancer and hematopoietic diseases paves the way for aspects of the present invention to provide the use of materials and methods, such as are disclosed and discussed above, for

establishing the presence or absence in a test sample of a variant form of the gene, in particular an allele or variant specifically associated with cancer or hematopoietic diseases. This may be for diagnosing a predisposition of an individual to cancer or hematopoietic disease. It may be for diagnosing cancer or hematopoietic disease in a

5   patient with the disease as being associated with an altered or aberrantly regulated *PPT-I* gene.

This allows for planning of appropriate therapeutic and/or prophylactic measures, permitting stream-lining of diagnosis, treatment and outcome assessments. The approach further stream-lines treatment by targeting those patients most likely to

10   benefit.

According to another aspect of the invention, methods of screening drugs for therapy, i.e., for restoring or inhibiting PPT-I product functions are provided.

The *PPT-I* polynucleotides and fragments can be employed in drug screening assays, and may either be free in solution, affixed to a solid support or within a cell.

15   One method of drug screening utilizes a *PPT-I* promoter/reporter gene construct, preferably in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays. One may determine, for example, formation of complexes between a *PPT-I* promoter and the agent being tested, or examine the degree to which the formation of a complex between a PPT-I polypeptide or fragment

20   and a known ligand is interfered with by the agent being tested.

In a particularly preferred embodiment of the invention, the promoter region or 3' region of the *PPT-I* gene is operatively linked to a reporter gene. Reporter genes suitable for this purpose include, without limitation, beta galactosidase, luciferase, chloramphenicol acetyltransferase, and green fluorescent protein. Methods for

25   operably linking the coding regions for the reporter genes to the *PPT-I* regulatory sequences are well known to those of ordinary skill in the art.

Following introduction of such DNA constructs into recipient host cells, the cells may be contacted with agents suspected of affecting expression of the reporter gene. Agents capable of altering expression of the reporter gene may prove

30   efficacious in regulating *PPT-I* expression, thereby having therapeutic advantage in the treatment of cancer or other disorders where altered *PPT-I* expression plays a role.

Pharmaceuticals and Peptide Therapies

The discovery that *PPT-I* gene expression is altered in cancer or hematopoietic diseases facilitates the development of pharmaceutical compositions useful for treatment and diagnosis of these syndromes and conditions. These

5   compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral,

10   intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the

15   case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual.

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

20

EXAMPLES

Materials and Methods Utilized in the Examples

25   Goat antihuman (h) SCF, antihIL6, SCF and IL6 were purchased from R&D Systems (Minneapolis, MN). Rabbit antihILla and rabbit antiSP were purchased from Endogen (Boston, MA) and Amel Products Co., Inc. (NY, NY) respectively. Alkaline phosphatase (AP)conjugated goat antirabbit IgG and AP goat antimouse IgG were purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD).

30   APconjugated swine antigoat IgG was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Hoffman LaRoche (Nutley, NJ) provided rhIL1∝.

Reagents

For purposes of the present invention, Substance P, streptavidin and bovine serum albumin (BSA) may be purchased from readily available commercial sources such as Sigma (St. Louis, MO). Substrate for alkaline phosphatase, 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BLIP/NBT) was obtained from Kirkegaard and Perry Laboratories. CP-96,345-1 is commercially available from sources such as Pfizer, Inc. (Groton, CT). SR 48968 is commercially available from sources such as Sanofi Recherche (Montpellier Cedex, France).

Quantitation of SP-IR

Competitive ELISA quantitated SP-IR in supernatants from breast cancer cell cultures that were 80% confluent. Cell-free supernatants were stored in siliconized tubes at -70°C until ready to be assayed. Streptavidin, 100 ~ml at 5 g/ml in distilled water, was added to Immulon 96-well plates (Dynatech Laboratories Inc., Chantilly, VA). Streptavidin was allowed to dry at 37°C. After this, wells were blocked with 5% nonfat dry milk for 2 hr at room temperature and then washed with PBS containing 0.1% Tween-20 (PBST). Chiron Mimotopes (Emeryville, CA) synthesized biotinylated-SP, with spacer arm. Stock solution was diluted in 0.1% (v/v) acetic acid at 5 mg/ml, aliquoted in siliconized tubes and stored at -70°C. Working solution was diluted at 750 ng/ml with PBS containing 0.1 % (w/v) BSA and 0.1% (w/v) sodium azide. Biotinylated-SP (100 ml) was added to wells and plates incubated for 1 hr at room temperature. Plates were washed (x4) with PBST. Competition by the mobilized and soluble SP for anti-SP forms the basis for the next step. Equal volume (50 μl) of optimum rabbit anti-SP (1/15,000), and unknown or standard solution were added to wells. Plates were incubated at room temperature for 1 hr. Each unknown was assayed in triplicate as undiluted and three serial dilutions. Bound anti-SP was detected by incubating for 1 hr with optimum (150 ng/ml) AP-goat antirabbit IgG. Color was developed with Sigma 104 phosphatase substrate as described (Rameshwar, P. *et al.* (1996), *Blood* 88, 98-106). A standard curve was developed with O.D. (405 nm) versus 12 serial dilutions of standard SP that ranged from 100 to 0.08 ng/ml. Quadruplicate wells with controls included total (anti-SP and PBS) and background color (anti-SP omitted).

Quantitative RT-PCR

In one embodiment of the present invention, quantitative RT-PCR was performed with total RNA extracted from breast cancer cells. Total RNA (2 pg) was subjected to reverse transcription (RT) in 25 μl for 1 hr at 42° C. Following reverse transcription, the reaction was stopped at 94°C for 5 mins. RT mixture contained 50 mM Tris (pH 8.3), 30 mM KCl, 6 mM $MgCl_2$, 30 U M-MLV-RT (Boehringer Mannheim Biochemicals), 10 mM dithiothreitol, 40 U RNase inhibitor (Perkin Elmer-Cetus, Norwalk, CT), 5 μM random hexamer (Life Technologies, Grand Island, NY) and 3.0 mM dNTP (Boehringer Mannheim Biochemicals). Competitive PCR was performed in 50 μl volume with 200 ng unknown cDNA and various log-fold dilutions of standard DNA (10^{-2} 10^{-6} attomole/L). PCR reactions contained 20 mM Tris (pH 8.4), 10 mM KCl, 2 MM $MgCl_2$, 0.8 mM dNTP, 0.4 μM of each primer and 2.5 U Taq DNA Polymerase (Perkin ElmerCetus). Samples were overlaid with oil and then amplified for 35 cycles in a DNA thermal Cycler 480 (Perkin Elmer-Cetus). The profile for each cycle was 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. Reactions were subjected to a final extension at 72°C for 7 min. PCR products (10 μl) were separated by electrophoresis on 1.5% agarose containing ethidium bromide. Band intensities were quantitated with a Fluorimager (Molecular Dynamics, Sunnyvale, CA) and data analyzed with the ImageQuant software. Amplicon sizes were verified by comparison with either 1kb DNA ladder, DNA/Hind III fragments or low DNA mass ladder, all purchased from Life Technologies. DNA standards were prepared with PCR MIMIC construction kit, purchased from Clontech (Palo Alto, CA). Gene-specific nucleotides were synthesized at the Molecular Resource Facility, UMDNJ-New Jersey Medical School (Newark, NJ) with an ABI model 392 DNA/RNA synthesizer (ABI, Foster City, CA). Acceptable primers for *PPT-I* include, for example.

5'AAT TTA CCT GTC ATT GCC C3' (sense) (SEQ ID NO:8) and

5-AGC CCT TTG AGC ATC TTC 3 (antisense) (SEQ ID NO:9) span

Exons 3 and 7 of α-, β and γ-PPT-I with sizes equivalent to 261 bp, 315 by and 270 by respectively (Harmar, A.J. *et al., FEBS Lett.* 208, 67-72). *PPT-I* standard of 157 base pairs in length was constructed with the following: primer pair, 20 gene-specific

obtaining informed consent. Bone marrow cells were cultured in 25 cmz tissue culture flasks (Falcon 3109, Becton Dickinson Labware, Lincoln Park, NJ) at 33°C for 3 days. After three days, granulocytes and red cells were removed by FicollHypaque (Sigma) density gradient. The mononuclear fraction was replaced into

5   flasks. Cultures were reincubated until confluence with weekly replacement of 50% stromal medium.

Stimulation of bone marrow stroma

Transfected bone marrow stroma was stimulated with optimal concentrations

10   of IL-1α (2.5 ng/ml), SCF (8 ng/ml) or 1 nM substance P, SP (Sigma, St Louis, MO) and/or 10 nM NK-1-specific antagonist (CP-99,994) in α-MEM with 2% FCS. In other embodiments, bone marrow stroma was stimulated with 5μg/ml forskolin, FK (Sigma) for 2 and 5 h. SCF was purchased from R&D Systems (Minneapolis, MN) and IL-1α was obtained from Hoffman LaRoche, Nutley, NJ. Pfizer, Inc. (Groton,

15   CT) provided CP-99,994. Substance P and CP-99,994 were dissolved and stored as described in Ramewshwar *et al* (1996), *supra.* Optimal parameters were determined with dose-response and time-course studies.

Isolation of Poly A RNA

20   Confluent bone marrow stroma was stimulated with 25 ng/ml rhIL-1α or 10 ng/ml SCF for 24 h. Poly A RNA was isolated from total RNA by selection (2x) on oligo-dT cellulose (Life Technologies). The cellulose column was washed once with 10 mM Tris, pH 7.0; 0.5 M NaCl; 1M EDTA and once with 10 mM Tris, pH 7.0; 0.2 M NaCl; 1M EDTA. Poly A RNA was eluted with 10 mM Tris (pH 7.0) and 1M

25   EDTA. RNA was precipitated by standard techniques and then resuspended in DEPC-treated water. The purity was verified by the absence of rRNA.

***In vitro* translation**

In vitro translation for *PPT-I* was performed with poly A RNA from stroma

30   stimulated with IL-1a or SCF (Rameshwar, P., *et al., Leuk. Lymphoma 18,* 1-10; Merrill, J.E. *et al., FASEB J. 9,* 611-618). As verified by quantitative RT-PCR, IL-1α and SCF induced 250,100 ± 980 (n=6) and 134,340 ± 545 (n=6) molecules of *β-PPT-*

I/μg poly A, respectively. We verified the presence of IL-la, IL6 and SCF mRNA in each preparation of poly A RNA (n=3) by northern analysis. Translation reactions were performed in siliconized tubes with 50 μl of the following: rabbit reticulate lysate system (Promega, Madison, WI), 0.5 μg poly A RNA, 20 μM Met-free amino

5   acid mixture (Promega) and 40 μCi L [^{35}S] Met (>1, 000 Ci/mmol, Dupont/New England Nuclear). Reactions were incubated for 16 hr at 30°C. Parallel reactions contained cytosolic extracts from breast cancer cell lines or MCF12A. Cytosolic extracts were prepared from 10' cells in a final volume of 0.5 ml as described in Rameshwar, P. *et al.*, *Immunol.* 153, 2819-2830. Positive reactions contained

10   Luciferase RNA (Promega). Background/negative reactions contained water instead of poly A RNA.

Quantitation of SP-IR, SCF, IL-6 and IL-1α was performed by ELISA using 2 μl of reaction mixture. The remaining mixture was used for immunoprecipitation of SP. Proteins were precipitated in the 2 μl sample with trichloroacetic acid (Sigma).

15   Precipitates were pelletted, washed (5x) with PBS, dried, redissolved in 10 μl of sterile distilled water and then quantitated for total proteins using a microassay (BioRad, Hercules, CA). SP-IR levels are expressed per μg poly A RNA.

Immunoprecipitation and Western blot

20   Immunoprecipitation and western blots were performed as described (Rameshwar *et al.* 1994, *supra*). Briefly, equivalent protein (1 μg) from each translational reaction was incubated at 4°C overnight with anti-SP (1/15,000), anti-hSCF (1 ng/ml), anti-IL-lα (1 mg/ml) or anti-hIL-6 (1 mg/ml). Control reactions were incubated with nonimmune species-specific IgG. Immune complexes were

25   selected by incubating at 4°C for 6 hr with protein A sepharose CL 4B (Sigma). Following incubation with protein A, centrifugation of the sepharose was performed at 4°C for 30 min at 10,000 g. Pellets were washed with PBS, resuspended in sample buffer and then electrophoresed on 16% SDS-PAGE. Positive control lanes contained SP, IL-1α or SCF. Proteins were electrophoretically transferred to Immobilon-P

30   membranes (Millipore, Bedford, MA) and then incubated with anti-SP (1/15,000), anti-SCF (1 ng/ml), anti-IL-6 (1 mg/ml) or anti-IL-lα (1 mg/ml). Membranes were washed and then incubated with the appropriate AP-conjugated second antibody (50

ng/ml). Color was developed with BCIP/NBT. The M, of developed bands was compared with prestained low range protein standards (Diversified Biotech, Newton Centre, MA).

DNA-binding proteins were extracted from transfected stromal cells using a
5   rapid micro preparation technique as described (Andrews, N.D. et al., 1997, *Nuc. Acids Res* 19:2499). Protein extraction for endogenous CRE-binding proteins were performed by boiling for 5 – 10 mins in 100 mM Tris and 4% SDS. Protein concentrations were determined using BioRad DC protein assay. Proteins were separated on 15% SDS-PAGE and then transferred to PVDF transfer membrane
10   (NEN). Membranes were incubated overnight with rabbit anti-CREM, anti-phospho-CREB (Cell Signaling, Beverly, MA) or anti-CREB (Cell Signaling). The working dilutions of antibodies were at 1/1000. Anti-CREM cross-reacts with the different isoforms of CREM proteins and ICER. At the end of the incubation period with the primary antibody, membranes were washed and then incubated with HRP-conjugated
15   goat anti-rabbit IgG (1/5000) for 45 mins. HRP was developed with ECL western blotting detection reagents (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

Transfection and reporter gene assay

pGL3-basic with inserts of different fragments from *PPT-I*-p1.2 was co-
20   transfected with pβ-gal-Control (0.5 µg each) in 80% confluent bone marrow stroma using SuperFect (Qiagen, Valencia, CA). After 48 h cells were scraped in 30 µl 250 mM Tris (pH 8.0) and then lysed by freezing and thawing in a dry ice/ethanol bath. Cell-free lysates (24 µl) were obtained by centrifugation at 15,000 *g* for 5 min at 4°C and then diluted with 5x cell culture lysis buffer (Promega). Luciferase and β-gal
25   activities were quantitated with 10 µl of lysates using Luciferase assay system (Promega) and Luminescent β-galactosidase detection kit II (Clontech) respectively. In the experimental model, the ratios of Luciferase/β-gal in cells transfected with vector alone ranged from 0.18 to 0.19 and was normalized to 1. Because cytokines induce the promoter upstream of β-gal, in cytokine-stimulated cells, luciferase
30   activity was presented/mg of total protein the levels normalized with stroma transfected with vector alone. Total protein was determined using standard techniquees well known in the art.

CRE and CRE-like mutation

Mutations were performed with a mutagenesis kit (Stratagene, La Jolla, CA). The desired mutant sequences (Table 1) were synthesized within 40 nt in the forward and reverse directions and then used in PCR with pGL3 containing wild type Upstream/N0. After PCR amplification, mutation was verified by identification of *Apa* L1 within CRE-like mutant and the loss of *Xho* 1 in CRE mutant.

Electrophoretic Mobility Shift Assay

Mutant or wild type CRE and CRE-like sequences, 20 ng (Table 1) were end labeled with γ-^{32}P-ATP using T4 polynucleotide kinase. Labeled probe was incubated with 2 μg of CREMτ or ICERIIγ in the presence or absence of excess cold competitor for 1 h. Reactions were separated on 4% PAGE, which were dried and then developed by autoradiography after 24 h.

In situ hybridization

In situ hybridization was performed as described (Rameshwar, P. *et al.* (1998), *Am. J. Hematol.* 59, 133-142). For each transcript, hybridization was performed with a cocktail of three antisense biotinylated oligonucleotides, each 18 nucleotides in length. Suitable oligonucleotides include, but are not limited to, *NK-1*, nucleotides 67-84, 439-456 and 815-832; *NK-2*, nucleotides 151-168, 712-729 and 1001-1018; β-*PPT-I*, nucleotides 210-227, 350-367 and 429-446.

Paraffin sections, 4 μm, from breast biopsies were placed on Superfrost/Plus slides (Fisher Scientific, Springfield, NJ). Sections were deparaffinized by the following sequential steps: 56°C overnight, xylene (2x5 min), 99% ethanol (2x1 min), 95% ethanol (2x1 min) and DEPC-treated water (1x5 min). All incubations were performed at room temperature. This was followed by rehydration in 2x SSC at 37°C for 30 min. Sections were washed with DEPC-treated water for 5 min at room temperature and then incubated with 30 μg/ml proteinase K for 1 hr at 37°C. Negative control slides were incubated with 100 μg/ml RNase for 30 min at 37°C. Enzyme activities were stopped with 0.4% paraformaldehyde in PBS. Cells were prehybridized at 37°C for 1 hr in equal volumes of prehybridization solution (5 Prime

3 Prime, Boulder, CO) and formamide, and 10 mg/ml salmon sperm (5 Prime 3 Prime). Cells were hybridized at 37°C for 24 hr with 200 ng/ml of oligonucleotide cocktail. Following hybridization, sections were washed sequentially for 5 min at 37°C in the following buffers: 4x SSC/30% formamide, 2x SSC/30% formamide and

5   0.2x SSC/30% formamide. Sections were next incubated for 1 hr at room temperature with 1.25 μg/ml avidinAP (Boehringer Mannheim Biochemicals). Control slides were incubated with a cocktail of sense oligomers. AP was developed with BLIP/NBT. Slides were counterstained with Harris Modified Hematoxylin (Fisher Scientific) and then examined with an Olympus Bx40 microscope (New

10   Jersey Scientific, Inc., Middlebush, NJ).

In situ hybridization for the luciferase reporter vector, pGL3 (Promega, Madison, WI) was performed with a 300 bp ampicillin DNA probe, which was labeled with a random biotin labeling kit (NEN, Boston, MA). Probe was prepared by PCR with primers specific for ampicillin gene and pSEAP2 (Clontech, Palo Alto, CA)

15   as template. The second labeling was performed with antibodies for the three major stromal subsets as described. Primary antibodies for fibroblast, endothelial cells and macrophages were specific for prolyl 4-hydroxylase (Dako, Carpinteria, CA), von Willebrand factor (Dako) and CD14. After this, cells were incubated for 30 mins with rat PE-conjugated anti-κ (Becton Dickinson Immunocytometry Systems, San Jose,

20   CA) and FITC-avidin (Vector Laboratories, Burlingame, CA). Cells were examined for fluorescence intensity with excitation at 495nm/emission at 515 nm for FITC and excitation at 595 nm/emission at 606 nm for PE.

Cell proliferation

25   Clonogenic assays studied the proliferation of breast cancer cell lines. Duplicate cultures were performed with 10^3 cells/ml in 1.2% methylcellulose matrix that contained various concentrations (between about 100 to 0.001 mM) of NK-1, NK-2 or (NK-1 + NK-2) antagonists. Baseline cultures contained media alone. For each cell line, the assay contained the appropriate culture media. Cultures were

30   incubated for 2 wk at 37°C. This was followed by enumeration of colonies that contained more than 10 cells. In a highly preferred embodiment, the concentration of antagonist is 1 nM.

Statistical analysis

Data were analyzed using analysis of variance and Tukey-Kramer multiple comparisons test and the Students ttest to determine the significance (P value) between experimental values. A P value of <0.05 was considered significant.

5

Example 1.

Expression of *PPT-I, NK-1* and *NK-2* in Breast Cancer cells

We studied *PPT-I, NK-1* and *NK-2* expression in breast cancer cell lines and breast biopsies. In the initial studies, we determined the production of SP-R by breast

10   cancer cells, nontransformed mammary epithelial cell lines and unrelated cell lines, CCL64, MDBK and L929. As shown in Table 1, SP levels were more than 7 fold higher in breast cancer cells than nontransformed mammary epithelial cells and SP was not detected in the culture media of the unrelated cell lines. As Substance P is the major *PPT-I* peptide product, its high levels prompted us to determine whether

15   enhanced production of SP correlated with steady state *PPT-I* mRNA using quantitative RT-PCR.

Table 1.
Production of immunoreactiveSP (SP-IR) by breast cancer cell lines.

Cell Lines	SP-IR (ng/ml)
Transformed	
MDAMB330	146 + 5
T47D	209 + 38
ZR7530	159 + 12
BT474	160 + 16
DU4475	90 + 3
BT 483	102 + 8
184B5	64 + 8
Nontransformed	
MCF12A	9 + 2
Hs578Bst	8 + 1
Other	
CCL64	<0.08
L929	<0.08
MDBK	<0.08

20
25
30
35

ELISA quantitated SP-R in the culture media of breast cancer cell lines. Details of the technique are described in above. Each point is the mean (+SD) of 8 different cell passages.

40

The *PPT-I* gene consists of seven exons that can be alternately spliced into four transcripts (Harmar, A.J. *et al.*, *supra*). Since each *PPT-I* transcript contains exons 3 and 6 (Harmar, A.J. *et al.*, *supra*), we designed oligonucleotide primers that span these two exons. In each RT-PCR reaction, we observed single fragments

5   equivalent to 315 base pairs, consistent with *PPT-I* (Harmar, A.J. *et al.*, *supra*). The average levels of *β-PPT-I* in the breast cancer cell lines were 53 molecules/g total RNA, as shown in Table 2. As also described in Table 2, nontransformed mammary epithelial cells showed undetectable *PPT-I*, <1 molecule/g total RNA. The results indicate that *PPT-I* is highly expressed in breast cancer cell lines and that the

10   predominant transcript is β-PPT-I.

We next determined if the receptors for PPT-I peptides were also increased. As shown in Table 2, quantitative RT-PCR *NK-1* and *NK-2* mRNA levels were increased in malignant cells. *NK-1* mRNA was undetected in nontransformed mammary epithelial cells, but *NK-2* mRNA was elevated (Table 2). Table 2 also describes that in

15   four breast cancer cell lines, *NK-2* levels were significantly ($p \leq 0.5$) less than MCF12A and Hs578Bst cells. In other words, in contrast to nontransformed mammary epithelial cells which show an increase in *NK-2* mRNA alone, breast cancer cells have significantly high levels of *PPT-I*, *NK-1* and *NK-2* mRNA ($p < 0.05$).

Table 2

20   **Levels of *β-PPT-I*, *NK-1* and *NK-2* mRNA in Breast Cancer cell lines.**

Cell Lines	β-PPT-I	NK-1	NK-2
		(molecules/µg total RNA)	
Transformed			
25 MDAMB330	45±3	1686±52	4740±40
T47D	65±2	2600±48	3768±30
ZR7530	42±1	25+2	1218+25*
BT474	57±4	28±6	429±22*
DU4475	55±3	38±8	389±20*
30 BT 483	62±5	1240±35	560±25*
Nontransformed			
MCF12A	<1	<1	2419±28
Hs5782st	<1	<1	2225±44

35   Levels of *β-PPT-I*, *NK-1* and *NK-2* mRNA were determined by quantitative RT-PCR using total RNA (2 pg) from malignant or nontransformed mammary epithelial cells. For each cell line, quantitations were performed with RNA extracted from ten different passages. *p<0.05 vs. nontransformed cells

Example 2.

Expression of PPT-I, NK-1 and NK-2 in breast biopsies

Because cell lines undergo multiple passages, we next determined whether

5   *PPT-I, NK-1* and *NK-2* are similarly expressed in malignant breast biopsies.

Comparison was made with benign tissue. By *in situ* hybridization, we determined

that *PPT-I, NK-1* and *NK-2* are expressed in all malignant tissues. In benign tissues,

PPT-I and *NK-1* mRNA were not detected, whereas *NK-2* mRNA was dense in

benign tissue. No signal was detected with breast tissues (malignant and benign)

10   hybridized with sense oligonucleotides. Parallel immunohistochemical studies

indicated that malignant tissues were positive for SP-R and negative in benign tissues.

Qualitative observations of the signals presented by *in situ* hybridization and the data

shown for breast cancer cell lines in Tables 2 and 3 indicate comparable expression of

PPT-I, NK-1 and *NK-2* in malignant breast biopsies and breast cancer cell lines.

15

Example 3.

Translational rate of β-PPT-I mRNA

Although increased, the levels of *PPT-I* mRNA in breast cancer cells (Table

2) could not explain the high SP levels (Table 1). We next investigated if the increase

20   in *SP-R* may be due to increased translation of *β-PPT-I* using an *in vitro* assay that

contained soluble cytosolic extracts from either breast cancer cells or MCF12A.

Since IL-1 induces PPT-I in bone marrow stroma (Ramewshar, P. (1997), *supra*;

Merrill, J.E., *supra*), poly A RNA from IL-1 stimulated bone marrow stroma was used

as *PPT-I* mRNAcontaining substrate. Before each assay, we verified the level of

25   *PPT-I* mRNA in quantitative RT-PCR. Since each *PPT-I* transcript can produce

Substance P (Harmar, A.J. *et al.*, *supra*), its level was used as the read out to study the

rate of *PPT-I* mRNA translation. In six different experiments (±SD), we found that

SP-IR was increased 40±3 and 30±2 fold more in the presence of cytosolic extracts

from breast cancer cells than extracts from MCF12A (Table 3).

30

Table 3.
ImmunoreactiveSP (SP-IR) following in vitro translation.

Cytosolic Extracts	SP-IR	ILlα	IL6	SCF
				(pg/μg poly A RNA)
Transformed				
MDAMB330	3146±45	20±6	230±22	6±1
T47D	2750±28	10±2	435±25	5±2
ZR7530	3590±42	5±1	342±34	8±2
BT474	3650±36	12±3	156±21	2±1
DU4475	2990±30	8±3	332±18	10±3
Nontransformed				
MCF12A	75±20	15±5	12±4	5±2
Hs578Bst	65±5	18±3	26±6	4±2

In vitro translation was performed with *PPT-I*-containing poly A RNA in the presence or absence of cytosolic extracts from mammary epithelial cells. Aliquots, 2 1, were TCA-precipitated and then determined for SP-IR by ELISA. The data is represented as protein levels/g poly A. Details of the technique are described in above. For SP-IR, each point is the mean (+SD) of six different experiments. For ILlα, IL6 and SCF, each point is the mean (±SD) of three different experiments. Each experiment was performed with poly A RNA isolated from bone marrow stroma from a different donor and cell extracts from a different cell passage.

To further confirm that Substance P is present in the translation reactions, we performed immunoreactive techniques. We used equivalent quantities of proteins to immunoprecipitate Substance P. Immune complexes were analyzed in western blots to determine whether the precipitates were consistent with the predicted size of Substance P. In reactions without cytosolic extract, a light band was developed Cytosolic extracts from breast cancer cells showed strong bands. In contrast, no band was visible in reactions performed with extracts from normal mammary epithelial cells, MCF12A.

We next determined if the putative translational factors in the cytoplasm of breast cancer cells were unique to PPT-I mRNA. We studied the translation rate for IL-lα, IL-6 and SCF. For SCF, we used poly A RNA from IL-lα-stimulated stroma because this cytokine is expected to induce SCF. For IL-lα and IL-6, we isolated poly A RNA from SCFstimulated stroma. Immune complexes, analyzed in western blots showed no band for IL-lα and SCF. However, translation was increased for IL-6. ELISA was utilized to quantitate these results (Table 3). The results described in this

section show that cytosolic extracts from breast cancer cells increase SP-IR and IL-6 in an *in vitro* translation system. No effect was observed for SCF and IL-la.

Example 4.

5   **Role of PPT-I peptides in the proliferation of Breast Cancer cells**

Increased expression of *PPT-I* and *NK-1* in breast cancer cells (Tables 1 and 2) led to the next set of experiments. A functional role for these genes in breast cancer is established by determining whether PPT-I peptides, endogenously produced by breast cancer cells, can induce their proliferation through autocrine and/or

10   paracrine mechanisms. This question was addressed in six different experiments using clonogenic assay. Dose-response and timecourse studies indicated that the optimal parameters are 1 nM antagonist and two wk of incubation. Assays performed with various concentrations of cells showed distinct differences among various treatments if the cultures were performed with 10^3 cells/ml. NK-1 or NK-2

15   antagonists blunted breast cancer cell proliferation by approximately 40% when compared to cultures with media alone (Table 4). As seen in Table 4, cells cultured with both antagonists blunted cell proliferation by 20%. There was no significant difference (p>0.5) in cell proliferation when normal mammary epithelial cells (MCF12A and H5578Bst) were cultured with the antagonists. The data indicate that

20   alone, NK-1 and NK-2 antagonists blunted the proliferation of breast cancer cell lines and together, they exert more potent inhibitory effects. Reduced colony formation was not due to necrosis since >99% of the cells were trypan blue negative in suspension cultures, performed for two weeks with antagonists.

25   **Table 4**
Effects of NK Receptor antagonists on the
<u>**proliferation of breast cancer cell line**</u>

Cell lines	Media	CP96,345	SR48968	CP96,345 + SR 48968
		# of colonies /10^3 cells (±SD)		
Transformed				
T47D (20%)	150±5	60±4 (40%)	65±7 (43%)	30±3
BT474 (190)	180±10	70±5 (39%)	65±8 (36%)	34±6

ZR7530 (21%)	165±5	68±8 (41%)	60±10 (36%)	35±5
MDAMB330 (22%)	170±8	65±5 (38%)	64±4 (38%)	38±8
5 Du4475 (19%)	160±8	63±6 (39%)	60±5 (38%)	30±6
NonTransformed				
MCF12A	210+12	198±18 (94%)	200±9 (95%)	220±13
10 104%)				
Hs578Bst 103%)	200±11	201±12 (1%)	210±11 (105%)	205±15

Cells, 10³, were cultured for 2 weeks in methyl cellulose matrix with 1 mM of CP
15 96,345, SR48968 or both antagonists. After two weeks, colonies were consisting of
greater than 10 cells were counted. The results are expressed as the mean of four
different experiments (+SD). Each experimental time point is the average of duplicate
cultures. The percentage of cells cultured in media alone is shown in parentheses.

20 **Example 5.**

Sequences of the human *PPT-I*, *NK-2* and *SP-R* promoters, and the *PPT-I* 3'end.

The entire sequence of human *PPT-I* promoter has been cloned and is set forth
herein as SEQ ID NO:1. Cloning and analysis of this promoter are described in the
25 examples below. The sequences of the *NK-2* and *SP-R* promoters are set forth as SEQ
ID NOS: 2, and 3. As mentioned previously, each of these promoter sequences can be
operably linked to reporter genes to assess agents which influence expression of *PPT-I*,
NK-2 and *SP-R*. Accordingly, one aspect of the invention encompasses methods to
assess such agents.

30

SEQ ID NO:1

```
-740  CTATAGGGCA CGCGTGGTCG ACGGCCCGGC TGGTAAATTC CCCTTTCTCC  -691
-690  AAAATGTAAA ATAAATCTGC TTCCATCTTC TAAAATACTA TGGGACTAAA  -641
-640  CATCCTTTTG TTATGCTAAG GAAAAGCCAG TATTCGCGTT GATTTAGAAG  -591
-590  AGGGATGTTC TGGTTATAGA ACGATGCTGT GTCTCAGAAA CACTTAAATA  -541
-540  CTATTAAGCT AGAAATAGAA GGGAAAATAA TGCTTCCCCG CATCTCCCCT  -491
-490  CAAGTGTAGT CCTCTTTTTT TAGCCTGATT TCCGACGAAA TGTCTGAATG  -441
-440  CCTACAGTTA TTTGGCCATC CTGAAAAGTG CAACTTATCC TGACGTCTCG  -391
-390  AGGGACGGAA AAGTTACCGA AGTCCAAGGA ATGAGTCACT TTGCTCAAAT  -341
-340  TTGATGAGTA ATATCAGGTG TCATGAAACC CAGTTTCGAA GGAGAGGGGA  -291
```

```
-290   GGGGGCGTCA GATCTGCAGA CGGAAGCAGG CCGCTCCGGA TTGGATGGCG  -241
-240   AGACCTCGAT TTTCCTAAAA TTGCGTCATT TAGAACCCAA TTGGGTCCAG  -191
-190   ATGTTATGGG CATCGACGAG TTACCGTCTC GGAAACTCTC AATCACGCAA  -141
-140   GCGAAAGGAG AGGAGGCGGC TAATTAAATA TTGAGCAGAA AGTCGCGTGG  -91
-90    GGAGAATGTC ACGTGGGTCT GGAGGCTCAA GGAGGCTGGG ATAAATACCG  -41
-40    CAAGGCACTG AGCAGGCGAA AGAGCGCGCT CGGACCTCCT TTCCCGGCGG  +10
                                                          Exon 1


+11    CAGCTACCGA GAGTGCGGAG CGACCAGCGT GCGCTCGGAG AACCAGAGAA  +60
+61    CTCAGCACCC CGCGGGACTG TCCGTCGCAG TAAGTGCCCG CGCGGTGCTG  +90
                                  Intron 1
+91    GCCGCGGCTG CCCGGGTCAT CCCACCCCGC ATCTGTCCGA GGTGGCCGCG  +140
+141   CTGGGGGCGC CGCTGCGGCG AGGGACAGTG GGGAGACTGG CTTCCCAAAC  +190
+191   GCCAACGCCC CTCTTTGTCT TCCACCTGCA GAGTTTCCTG GTTTGAAGGT  +240
+241   GTGGGTTGGT GGGTTAGGGG GCTGGGGGAG CTGGGATTCA GGGAGAAGAG  +290
+291   GGTTGGAGAA TCTTTGGGAC GCGATTCTCT CGCCTAACCG GTACAGGTGA  +340
+341   GACTTCAGTC CTTATGTTTT TGATCTTGGT TCATCCGTTG TGGGGCAGAA  +390
+391   AATTCTGTTG CTTTAACTCT TGGATAACCA CCCCTAATAG ATACATTATT  +440
+441   TCTCTCTTTG GTGTCTTCTC CTCCTACCCC TTCCCAGAAA TCCGACATGA  +490
                                                          Exon 2
+491   AAATCCTCGT GGCCTTGGCA TCTTTTTTTC TGACGG
```

SEQ ID NO: 2: Upstream sequences and exon 1 of the NK-2 receptor. The TATA box is at bases 369-376. Genbank Accession No. M75101.

5' Untranslated region -Upstream of Exon I, NK-2

```
1    CGACGGCCCT GGCTGGTACT GCTACTGTTG CCGCCACCAA CAGAGATCAA
51   AGGCAGAGAC CCTTCTGCTA GGGTCCAAAG TCCAAACAGG CCACTCCAGA
101  GAGGAAACAG GCACACAGGC ACACACCCAC GGGAGGAGTA GGGGCCCAGG
151  AAGCACTCCC TCCCCAAGGG CAAGGATGGG GTTCCCATTC CACCCAGCAC
201  ATGCTCCTCA CATCTGCACA GCAGGGAGAC CAAACAATAG ATACAATTTC
251  AGTGCCTGAT TGTCGATCAA CTTACCCAGA AGTTCATAAT CCGAAAAATC
301  CATAAAGAAG CTCTTTCAAT TTCAGCATGT TTAAGTTTCA TGACTTATGG
351  TTTAGTGTTG TTTTTATATT GGATTCCATG GGTGGCATAA TCTTTTCAGC
401  ACTAGAGACC TTTAAAGGTC TTTCTCAGCT CACCCCGGGA GACAAGGGCT
451  GGGTGTCAGG AAAGTGACAC ACAGGGAGAA GCAGAAAATG GACTGGGAGT
501  GTGGGGGCCG AGGCCCAGCC ACGAGAAACC CAGGCGGTGC AAGGCAGAGC
551  CCTGGGAGCA CAGAGGCTGC TGTGCCGTGG GTTGCTGGTG AATGAGAAGC
601  CTCCTCTGCT TTAATGAAGA ACATGCCCCC CCCGACTCCC GCTAATCCTG
```

```
 651   CCCTGCCTTC ATGATCCACA CACCACAGGT GTGCACAGGT TCATGCGTGT
 701   GTGTGAGCTT AACACGTCAG CCGCACATAC AGTTGCACAG AAACATCTTC
 751   ACTGCTTTCA CACACGTGCA CACAGTCAAA TGACCAGGAG CAGGATCTTG
 801   GGGCAAACCT AGAGCAGCTT CTCAGGAGTT AGAACTCCAG CTTTGCTGTG
 851   GTTCCCAGAA GAGCCCTGAC TTTGTCCTAA GACAGTGGTT CTCAAAGTGA
 901   AGTGCTGGCT CCAGCAGCAT CAGTATCACC TGGGAACTCG CTGGAAACGC
 951   TCCGGGTTCT GGCTTCTCCT CCTAGAGCGC CCAGAGCTGT GGGGTCCTCC
1001   CTTCGGGCCA GAAACTCCAA TCATAAGTTT CTATGTACCA ACCCCTGTGC
1051   TAAGTAGACT TTGTGCACAT TATCTCCATT TAAAATTTCA CAAATGTACT
1101   GTCAGATGCA CACCCATTTT TCTATACTTC TACAGATGGG GTAAGACAGA
1151   GCTCAGAAAG GTTAAGAGAC TTGCCTGGAG TCACCAAACC AGGCTCCAAC
1201   TCCTTCTGTA TTCAGAATCA CTCTTCAGAC GTAGCTCCTG TCCTGGGCTG
1251   AAAGTCAACA TCCGCCGAGA GCTGGGCCCT CTGTACCAGC CCCATCTCCC
1301   CCAAGTCTCT CCCTGCCTCT GCAGCCAGTC CTAAATCTTT CAAGAGACAA
1351   GGCCAAGCAG GGGGTGGGAC CAGGGGCGGG AGCCAAAGCC CCCCCTCGTG
1401   AGCAGGCAGC ACCTCTGCCA AGGCCCCCAC TGGCCCTGCC CCAGAGAACG
1451   GCAGGGAAGC TGCAGCGAGG GCTGGCAGCT GGCAGAGTCC TGAGCACCCA
1501   GCACCCAGCC CGGCTTGCAG CCCAAAGCCT GGAGAGAGGC TGCTGCGCCA
1551   TTGACCTGTG GACTCCAGAG ACTCCCGCTG TGCATTCCTC TGATCTGGAA
1601   GGTTTCCTGA ATTACGTGAC GAGAAACCTG GGTTCGAGTC CTAACTTGTC
1651   ACCAACGTTC CTGAGTGACC TGGGCTGGTC CCGTCCCCTT GGAATCTCTG
1701   TCTTCCATCT CTTCAGCGAA GGGGTTGATT TATAAGGGTG TTTTCTGCTC

                                    TATA signal

1751   TGACACTGTG ATTTGAATTC TGTGTTTCCA CATGATATTC GAGAAGTCTG
1801   GCCGGAAGGA TGGAATCTGA AATGACAATG GTTCTGGACT GGGCTTTGTG
1851   CTCAGCCCAG CTCATCTTTG CCTGAGACCT AGGAGTGGCC CCAGGCTCTC
1901   CTGATGTGCC ACCACGCTTG GCATCTGCTC CTCTCCCTGC CCCCATATTC
1951   CCATGCTCTG AAGGGGAGTT CTCTTTCATA GCAAATCCGA GAGGAGCCGA
2001   GGAGCCAGGT CCTTTGTTCC AGACCCAGAA GCAGCCATGG GGACCTGTGA

                                                    Exon 1

2051   CATTGTGACT GAAGCCAATA TCTCATCTGG CCCTGAGAGC AACACCACGG
2101   GCACCACAGC CTTCTCCATG CCCAGCTGGC AACTGGCACT GTGGGCCACA
2151   GCCTACCTGG CCCTGGTGCT GGTGGCCGTG ACGGGTAATG CCATCGTCAT
2201   CTGGATCATC CTGGCCCATC GGAGGATGCG CACAGTCACC AACTACTTCA
2251   TCGTCAATCT GGCGCTGGCT GACCTCTGCA TGGCTGCCTT CAATGCCGCC
2301   TTCAACTTTG TCTATGCCAG CCACAACATC TGGTACTTTG GCCGTGCCTT
2351   CTGCTACTTC CAGAACCTCT TCCCCATCAC AGCCATGTTT GTCAGCATCT
2401   ACTCCATGAC CGCCATTGCT GCCGACAG
```

SEQ ID NO: 3: Gene for Substance P receptor. Genbank Accession No: X65177. The TATA box is at bases 1339-1342.

```
   1   ggatccaatt tttgcccggc ataagtgtat agtaaatttc ccagccttaa agcacttccc
  61   gagagatgct ttgagcgctc gcggtaccag tgcgtaaacg ccgctccccg gctggcgcgg
 121   gtgtgcgcca actccaacct gcgcgcaagt ctgccggtgc gcgctccagt cccacagctc
 181   cgagtccccg cagtgaaagg aggggcggt gcaccggggt agatgggccc ctgaggactc
 241   ccggggttca gttttccgcg gctgccaaga gggccaagtt ggacagtggc agggtcctga
 301   agcagatcag caacaaccgc aagtgctcca gccccaggtc ctcagacacg gaggaaaacg
 361   acaagaggcg gacacacaac gtcttggaac gtcagaggag gaacgagctg aagcgcagct
 421   tttttgccct gcgtgaccag atccctgaat tggaaaacaa cgaaaaggcc cccaaggtag
 481   tgatcctcaa aaaagccacc gcctacatcc tgtccattca agcagacgag cacaagctca
 541   cctctgaaaa ggacttattg aggaaacgac gagaacagtt gaaacacaaa ctcgaacagc
 601   ttcgaaactc tggtgcataa actgacctaa ctcgaggagg agctggaatc tctcgtgaga
 661   gtaaggagaa cggttccttc tgacagaact gatgcgctgg aattaaaatg catgctcaaa
 721   gcctaacctc acaaccttgg ctggggcttt gggactgtaa gcttagagac tgtcacttcc
 781   caggtgaatc agctagccag gtaactgagc tagatatttt gtggggggtgt ttcctaaaca
 841   cagcctcagg aaagttgttt tcgggacacc tggaccaggg agtcgtcgcc tctggcttct
 901   cggtagctgg agcgcggccc ggagcgcggc gctggcacat cgcccccaca catgaccgtt
 961   tcccattgcc acaggcaagc cgcctctgca gagctgtctc agggctctgg gcttcattcc
1021   ctggaagttg attgtcctcc actccagctg tttcccaaat ccttccttcc tcccagcacc
1081   cctcgtgcaa cgacgattcc agctgcggac cgcatctgtg tcagttactt ccaagccacc
1141   tactgccccc tcgcggagtg cgtggggctc ccggctcgca gactcccacg gcaagtagca
1201   agcagcaaaa ggcgtggtag ctgcggcggt ggaatgagac agttgtcaac agctggcgca
1261   cgtgccgccg tgcgcaccgg gactggcgag tacgcagccc aggtactgcc ccttcccagt
1321   gacgtctctg caggggggtta taaaagcctc gtgcgcagct aactcgcgag ctgagcaacc
1381   cgaaccgaga ggtgcccgcg aaactgcagg cggcggcagc ggcagcaaaa gagaaggaaa
1441   aatctccagc tggatacgaa gctccagaat cctggccata ggctcagaac ttttacaggt
1501   cgcgctgcaa tgggcccca cttcgctcct aagtcctcac gcagcacagg ctttgcctt
1561   tccctgcgga ggaggagaa ataggagttg caggcagcag caggtgcata aatgcggggg
1621   atctcttgct tcctagaact gtgaccggtg gaatttcttt cccttttca gtttaccgca
1681   agagagatgc tgtctccaga cttctgaact caaacgtctc ctgaagcttg aaagtggagg
1741   aattcagagc caccgcgggc aggcgggcag tgcatccaga agcgtttata ttctgagcgc
1801   cagttcagct ttcaaaaaga gtgctgccca gaaaaagcct tccaccctcc tgtctggctt
1861   tagaaggacc ctgagcccca ggcgccagcc acaggactct gctgcagagg ggggttgtgt
1921   acagatagta gggctttacc gcctagcttc gaaatggata cgtcctccc ggtggactca
1981   gacctctccc caaacatctc cactaacacc tcggaaccca atcagttcgt gcaaccagcc
2041   tggcaaattg tcctttgggc agctgcctac acggtcattg tggtgacctc tgtggtgggc
2101   aacgtggtag tgatgtggat catcttagcc cacaaaagaa tgaggacagt gacgaactat
2161   tttctggtga acctggcctt cgcggaggcc tccatggctg cattcaatac agtggtgaac
```

```
2221 ttcacctatg ctgtccacaa cgaatggtac tacggcctgt tctactgcaa gttccacaac
2281 ttcttcccca tcgccgctgt cttcgccagt atctactcca tgacggctgt ggcctttgat
2341 aggtgagatt agcctttgtg aaaaggcgag aaagtgctca tagaggacca tggcattgct
2401 gtgaggtttg gaactgggtg gggtatgggt caagtggaag attggccact ctgagggttt
2461 ttttactgat ca
```

The 3' end of the PPT-I gene is set forth herein as **SEQ ID NO: 4**. This sequence includes the mRNA encoding ²-PPT-I.

```
1     gagagtgcgg agcgaccacg tgcgctcgga ggaaccagag aaactcagca ccccgcggga
61    ctgtccgtcg caaaatccaa catgaaaatc ctcgtggcct tggcagtctt ttttcttgtc
121   tccactcagc tgtttgcaga agaaatagga gccaatgatg atctgaatta ctggtccgac
181   tggtacgaca gcgaccagat caaggaggaa ctgccggagc cctttgagca tcttctgcag
241   agaatcgccc ggagacccaa gcctcagcag ttctttggat taatgggcaa acgggatgct
301   gattcctcaa ttgaaaaaca agtggccctg ttaaaggctc tttatggaca tggccagatc
361   tctcacaaaa gacataaaac agattccttt gttggactaa tgggcaaaag agctttaaat
421   tctgtggctt atgaaaggag tgcaatgcag aattatgaaa gaagacgtta ataaactacc
481   taacattatt tattcagctt catttgtgtc aatgggcaat gacaggtaaa ttaagacatg
541   cactatgagg aataattatt tatttaataa caattgttta gggttgaaaa ttcaaaaagt
601   gtttattttt catattgtgc caatatgtat tgtaaacatg tgttttaatt ccaatatgat
661   gactccctta aaatagaaat aagtggttat ttctcaacaa agcacagtgt taaatgaaat
721   tgtaaaacct gtcaatgata cagtccctaa agaaaaaaaa tcattgcttt gaagcagttg
781   tgtcagctac tgcggaaaag gaaggaaact cctgacagtc ttgtgctttt cctatttgtt
841   ttcatggtga aaatgtactg agattttggt attacactgt atttgtatct ctgaagcatg
901   tttcatgttt tgtgactata tagagatgtt tttaaaagtt tcaatgtgat tctaatgtct
961   tcatttcatt gtatgatgtg ttgtgatagc taacatttta aataaaagaa aaatatctt
1021  g
```

SEQ ID NO:14 is a variant of SEQ ID NO:1.

```
1     cgacggcccg gctggtaaat tcccctttct ccaaaatgta aaataaatct gcttccatct
61    tctaaaatac tatgggacta aacatccttt tgttatgcta aggaaaagcc agtattcgcg
121   ttgatttaga agagggatgt tctggttata gaacgatgct gtgtctcaga aacacttaaa
181   tactattaag ctagaaatag aagggaaaat aatgcttccc cgcatctccc ctcaagtgta
241   gtcctctttt tttagcctga tttccgacga aatgtctgaa tgcctacagt tatttggcca
301   tcctgaaaag tgcaacttat cctgacgtct cgagggacgg aaaagttacc gaagtccaag
361   gaatgagtca ctttgctcaa atttgatgag taatatcagg tgtcatgaaa cccagtttcg
421   aaggagaggg gaggggcgt cagatctgca gacggaagca ggccgctccg gattggatgg
```

```
481   cgagacctcg attttcctaa aattgcgtca tttagaaccc aattgggtcc agatgttatg

541   ggcatcgacg agttaccgtc tcggaaactc tcaatcacgc aagcgaaagg agaggaggcg

601   gctaattaaa tattgagcag aaagtcgcgt ggggagaatg tcacgtgggt ctggaggctc

661   aaggaggctg ggataaatac cgcaaggcac tgagcaggcg aaagagcgcg ctcggacctc

721   ctttcccggc ggcagctacc gagagtgcgg agcgaccagc gtgcgctcgg agaaccagag

781   aactcagcac cccgcgggac tgtccgtcgc agtaagtgcc cgcgcggtgc tggccgcggc

841   tgcccgggtc atcccacccc gcatctgtcc gaggtggccg cgctgggggc gccgctgcgg

901   cgagggacag tggggagact ggcttcccaa acgccaacgc ccctctttgt cttccacctg

961   cagagtttcc tggtttgaag gtgtgggttg gtgggttagg gggctggggg agctgggatt

1021  caggagaag agggttggag aatctttggg acgcgattct ctcgcctaac cggtacaggt

1081  gagacttcag tccttatgtt tttgatcttg gttcatccgt tgtggggcag aaaattctgt

1141 tgctttaact cttggataac cacccctaat agatacatta tttctctctt tggtgtcttc

1201  tcctcctacc ccttcccaga aatccgac
```

SEQ ID NO: 15 is the PPT-I cDNA (Genbank NM 013998)

```
1     gcgccgcaag gcactgagca ggcgaaagag cgcgctcgga cctccttccc ggcggcagct

61    accgagagtg cggagcgacc agcgtgcgct cggaggaacc agagaaactc agcaccccgc

121   gggactgtcc gtcgcaaaat ccaacatgaa aatcctcgtg ccttggcag tcttttttct

181   tgtctccact cagctgtttg cagaagaaat aggagccaat gatgatctga attactggtc

241   cgactggtac gacagcgacc agatcaagga ggaactgccg gagccctttg agcatcttct

301   gcagagaatc gcccggagac ccaagcctca gcagttcttt ggattaatgg caaacgggga

361   tgctggacat ggccagatct ctcacaaaat ggcttatgaa aggagtgcaa tgcagaatta

421   tgaaagaaga cgttaataaa ctacctaaca ttatttattc agcttcattt gtgtcaatgg

481   gcaatgacag gtaaattaag acatgcacta tgaggaataa ttatttattt aataacaatt

541   gtttggggtt gaaaattcaa aaagtgttta tttttcatat tgtgccaata tgtattgtaa

601   acatgtgttt taattccaat atgatgactc ccttaaaata gaaataagtg gttatttctc

661   aacaaagcac agtgttaaat gaaattgtaa aacctgtcaa tgatacagtc cctaaagaaa

721   aaaatcatt gctttgaagc agttgtgtca gctactgcgg aaaaggaagg aaactcctga

781   cagtcttgtg cttttcctat ttgtttttcat ggtgaaaatg tactgagatt ttggtattac

841   actgtatttg tatctctgaa gcatgtttca tgttttgtga ctatatagag atgtttttaa
```

```
901    aagtttcaat gtgattctaa tgtcttcatt tcattgtatg atgtgttgtg atagctaaca

961    ttttaaataa aagaaaaaat atcttgaaaa aaaaaaaaa aaa
```

5

Example 6.

Cloning of *PPT-I* promoter

    The human β-*PPT-I* sequence was used as a guide in nested PCR to clone

10   upstream relative to Exon 2. PCR was performed with templates from five human

genomic libraries (PromoterFinder DNA Walking Kit, Clontech). Outer (AP1) and

inner (AP2) adapter sequences linked to the 5′ ends and gene specific primers,

+124/+151 and + 88/+116 (Harmar, A.J. *et al.,* supra) were used in nested PCR.

Procedures were performed according to manufacturer's instructions. Single bands

15   from three libraries were cloned into pNoTA/T7 (5 Prime → 3 Prime, Boulder, CO).

The resulting DNA product was sequenced using techniques well known in the art. The

sequenced DNA indicated overlapping sequences. The cloned fragment was analyzed

using Wisconsin Package, Version 10 Genetics Computer Group, Inc., Madison, WI.

    PCR was used to sub-clone the sense and anti-sense orientations of PPT-I-p1.2

20   and the following fragments in pGL3-basic: **1.** −722 bp relative to Exon 1,

Upstream/N0, **2.** Upstream/N1, −722 Δ -589, **3.** Exon 1, **4.** Intron 1 and, **5.** Exon1/Intron

1. To obtain the desired orientation, PCR primers were synthesized with sequences for

Hind III and *Kpn* I in the desired location.

25   **Example 7.**

Identification of *PPT-I* promoter

    By genomic walk using nested PCR with primers specific for Exon 2 of β-*PPT-I*

cDNA, three overlapping sequences were isolated from different DNA libraries,

upstream of Exon 2, *PPT-I.* Figure 2 shows the sequence of the longest fragment

30   (1.225 kb, PPT-I-p 1.2). This sequence overlaps with Exon 1 of the cloned sequence of

β-*PPT-I* (Harmar, A.J. *et al., supra* This information was used together with computer-

assisted analyses to determine the location of the TAATA box. *PPT-I*-p1.2 consists of

the following: Exon 2 (+499, ATG and downstream sequences omitted), Intron 1

(+89/+498), Exon 1 (+1/+89) and -722 nt relative to Exon 1, hereafter referred as

"Upstream/N0".

To determine if *PPT-I-p1.2* contains promoter activity, we ligated the sense and anti-sense sequences in the reporter (luciferase) vector, pGL3-basic, and then quantitated reporter activity in transfected, primary human bone marrow stromal cells.

5 These cell types were chosen to study *PPT-I-p1.2* because they could be induced to express endogenous *PPT-I* (Kramer, M..S. *et al.* 1998, *Science* 281:1640) and would therefore contain necessary trans-acting factors to study *PPT-I* promoter. A second advantage of using stromal cells is that this strategy could examine the regulation of *PPT-I* in bone marrow stroma, the hematopoietic supporting cells (Mullersieburg, C.E.

10 *et al.* 1995. *Stem Cells* 13:477), thus providing insights into the role of this gene in hematopoiesis.

We first determined if the primary stromal cells could incorporate the transfected DNA. *In situ* hybridization for ampicillin vector showed that >80% of the stromal cells incorporated plasmid DNA. Co-labeling by immunofluorescence with

15 specific antibodies indicated that most of the plasmid DNA was taken up by two of the three major stromal subsets: macrophage and fibroblast. Cells transfected with *PPT-I-*p1.2 in the sense orientation resulted in 480±30 increase in normalized luciferase activity (Figure 3) and <0.8 fold in the anti-sense orientation (not shown). These results indicate that *PPT-I-*p1.2 contains promoter activity. In addition to bone marrow stromal

20 cells, we also observed promoter activity in two other types of cells, CCL64 and skin fibroblasts.

We next narrowed the region containing promoter activity by sub-cloning different fragments of *PPT-I-*p1.2 in the sense and anti-sense orientations in pGL3-basic. Figure 3 shows the ratio of luciferase/β-gal in stroma transfected with

25 Upstream/N0, Upstream/N1 (-722 Δ -589), Upstream/ N2 (-722 Δ -392), Upstream/N3 (-722 Δ -230), Exon 1, Exon 1/Intron 1 or Intron 1. There was no significant difference in luciferase activities in cells that were transfected with Upstream/N0 (36±2) and Upstream/N1 (30±2), *p*>0.5. Further deletion in the 5' region (Upstream/N2 and Upstream/N3) resulted in <2 normalized luciferase activity. Therefore, the 5' end of

30 Upstream/N0 contains of sequences that are important for promoter activity. The relative lack of promoter activity by Exon 1 and Intron 1 further supports the presence of a promoter in Upstream/N0 (Figure 3). In contrast to the individual sequences, Exon

1, placed in tandem with Intron 1, showed significant increase in luciferase activity, suggesting that these sequences contain a second, but weak promoter and/or regions that might be stabilizing the DNA. Taken as a whole, the results shown in Figure 3 indicate that *PPT-I-p1.2* has a strong (Upstream/N0) and possibly a weak (Exon

5 1/Intron 1) promoter. Regulatory regions in Exon 1/Intron 1 are interesting because the protein-coding region for each of the four *PPT-I* transcripts is within Exon 2 (Harmar, A.J. *et al., supra*).

Example 8.

10 **Characterization of CRE and CRE-like in Upstream/N0**

Computer analyses of Upstream/N0 indicated consensus sequences for two CRE that we termed CRE and CRE-like (Figure 2). We first established if these two sequences could bind CRE-binding proteins using ICERIIγ in gel shift assay (Molina, C.A. *et al*, 1993. *Cell* 75:875), Figure 4A. The results indicated that ICERIIγ binds to

15 wild-type CRE and CRE-like indicating that the latter could be a CRE site (Table 5). However, ICERIIγ did not bind to the mutants (Figure 4A) indicating that the particular mutation adequately prevents interaction with the specific proteins. These results justify the use of mutants in studies to determine the specificity of CRE and CRE-like in the analyses of *PPT-I* promoter.

20 **Table 5. Wild type and mutant cAMP response elements (CRE and CRE-like).**

Gene	Sequence	Homology
CRE-somatostatin	5'-TGACGTCT	
CRE-PPT-I (wild type)	5'-TGACGTCT	CREB, CRE
CRE-PPT-I (mutant)	5'-*A*GA*T*GT*T*T	
CRE-like-PPT-I	5'-TTGCGTCA	CREB, CRE, ATF
CRE-like-PPT-I (mutant)	5'-TTG*T*G*CAC*	

Italics and underlined sequences represent points of mutations.

30

Since the experimental model included transfection of bone marrow stroma to study CRE and CRE-like, we next determined if these cells express CRE-binding proteins and whether they could be phosphorylated by FK, a cAMP-inducing agent. Consistent with other cell types, the levels of CREM did not show any significant

35 change regardless of cell stimulation (Figure 4B: lanes 1-3, top arrow). However,

stromal cell stimulation with FK resulted in the presence of ICER after 2 h and an

increase by 5 h (Figure 4B, lanes 2 and 3, lower arrow). ICER was not detected in

unstimulated cells (Figure 4B, lane 1, lower arrow). Figure 4C showed constitutive

expression of CREB proteins. FK stimulation resulted in phosphorylation of CRE-

5   binding proteins (CREB, CREM and ATF): CREB and CREM co-migrated together,

(Figure 4D, top bands, lanes 2 and 3) and ATF-1, lower bands (Figure 4D, lanes 2 and

3). The results shown in Figures 4B to 4D showed that similar to cells from several

sources, bone marrow stroma expresses CRE-binding proteins.

          Before addressing the importance of CRE and/or CRE-like in the induction of

10   PPT-I by cytokines, we determined if these sequences are important for PPT-I

promoter activity using two different approaches. In the first approach, we co-

transfected bone marrow stroma with pGL3-Upstream/N0 and/or the transcription

factors that interact with CRE: CREMτ (activator) or ICERIIγ (repressor). Because

activation of CREM requires PKA phosphorylation (Molina,C., *supra*), we included

15   PKA-expression vectors. Cells were transfected with PKA+CREMτ, PKA or

PKA+CREMτ+ICERIIγ, and the levels of luciferase activities were quantitated. In the

second approach, CRE and/or CRE-like was mutated in Upstream/N0 and then co-

transfected. Western analysis confirmed the expression of CREMτ and ICERIIγ in the

transfected stromal cells (Figure 5A). The results of both approaches are shown in

20   Figure 5B. Co-transfection of pGL3-Upstream/N0 with wild type or mutant CRE

showed no change in luciferase activity (open bars). This demonstrated that sequences

other than CRE and CRE-like are involved in baseline promoter activity. Co-

transfection with PKA showed no change in luciferase activity in the wild type or single

mutant. Since PKA phosphorylates proteins other than those that bind to CRE sites, the

25   data shown for co-transfection with PKA and single mutants indicated that either one

CRE site could mediate optimal luciferase activity or that non-CRE sites are involved

in activation of the *PPT-I* promoter. There was significant reduction of luciferase

activity ($p<0.05$) when PKA was co-transfected with CRE double mutant. This

suggests that although other sites might be involved in the activation of *PPT-I*

30   promoter, an available CRE site is required for optimum activity given the appropriate

activation signal. The specificity of CRE-mediated responses is shown by the

significantly reduced activity of luciferase ($p<0.01$) when ICERIIγ was co-transfected

with PKA and CREMτ. Comparing luciferase activity in the co-transfectants of the double mutants with wild type CRE and CRE-like indicated that in the presence of the appropriate transcription factors, both CRE sites could contribute in either a synergistic or additive manner with other transcription factors to induce Upstream/N0. However, CRE and CRE-like binding factors demonstrated synergistic rather than additive effects.

Example 9.

Role of CRE and CRE-like in PPT-I induction: Model by IL-1α and SCF

Cytokines are important inducers of *PPT-I* (Ramewshwar, P. 1997, *supra*). We used two representative cytokines (IL-1 and SCF) to determine the physiologic significance for CRE and CRE-like in *PPT-I* regulation. These two cytokines were used because they induce *PPT-I* and the high affinity receptor for PPT-I peptides, neurokinin-1 (*NK-1R*) in bone marrow stroma, and they also activate the cAMP pathway. Table 6 shows the validity for using IL-1α and SCF in this model. Compared to unstimulated cells, IL-1α and SCF stimulation resulted in significant increase of endogenous *PPT-I* mRNA ($p<0.005$) and also modulate the mRNA for the receptors, NK-1 and NK-2 (Table 6). These observations are consistent with the modulation of NK-1 and NK-2 in bone marrow cells to regulate hematopoiesis (Rameshwar, P. 1997, *supra*; Rameshwar, P. *et al.* 1997,*supra*; Culman, J. *et al.* 1995, *Canadian J. Pharmacol.* 73, 885-891).

Table 6.
β-*PPT-I* and *NK-2* mRNA levels in SCF and IL-α-stimulated BM stroma

Stimuli	Molecules/µg total RNA		
	β-*PPT-I*	*NK-1*	*NK-2*
Unstimulated	16 ± 5	<10	2609 ± 70
SCF	1336 ± 134*	5253 ± 170*	273 ± 19*
IL-1α	4849 ± 121*	8106 ± 91*	968 ± 26*

Confluent BM stroma was stimulated with 8 ng/ml SCF or 2.5 ng/ml IL-1α for 16 h in serum-free α-MEM. Quantitative RT-PCR determined steady-state mRNA.
*$p<0.05$ vs. unstimulated, n = 10.

To address if SCF and IL-1a required CRE and CRE-like for *PPT-I* induction, we transfected bone marrow stroma with pGL3-Upstream/N0 with wild type or mutant CRE and/or CRE-like and then stimulated the transfectants with SCF or IL-1a. Compared to unstimulated cells, SCF and IL-1a stimulation showed 4- and 3-fold

5   increase in luciferase activities respectively (Figure 6A). SCF and IL-1a showed no significant induction of luciferase in cells transfected with the double mutants (Figure 6A). However, there was 1.5 fold less luciferase activity in the double mutants compared to cells transfected with wild type or single mutant (Figure 6A). These observations demonstrates that both CRE and CRE-like have roles in the activation of

10   Upstream/N0. Furthermore, the data suggest that with respect to *PPT-I* induction, the CRE sites are be the dominant regulatory regions for inducers that are associated with stimulation of the cAMP pathway and in the absence of CRE-binding proteins, cytokine could be repressors of *PPT-I* induction.

Generally, biological functions do not occur in a microenvironment with only

15   one stimulus. Therefore, we designed the next set of experiments with the aim of obtaining insights into the role of the two CRE in a microenvironment that is has have multiple soluble factors that are associated with the activation of cAMP pathway, such as cytokines and neurotrophic factors. Because CREM is activated by many cytokines, this protein was overexpressed as a model to mimic the presence of other stimuli that

20   could activate CRE-binding proteins. Stroma was co-transfected with pGL3-Upstream/N0 and CREMτ and then stimulated with IL-1α or SCF. For both cytokines, co-transfection with CREMτ resulted in significant induction of luciferase compared to cells transfected with pGL3-Upstream/N0 alone (Figure 6B). Luciferase induction by both cytokines was significantly reduced by co-transfection with ICERIIγ (Figure 6B).

25   These results supported an important role for CRE in *PPT-I* induction by at least two *PPT-I*-inducible cytokines that are associated with stimulation of cAMP pathway.

Example 10.

Autocrine activation of *PPT-I* through SCF-mediated expression of *NK-1*

30   In the next set of experiments, we investigated the possibility for an indirect mechanism in the activation of *PPT-I* promoter. We hypothesize that this pathway could occur independently, or in addition to other pathways through concomitant

induction of endogenous *PPT-I* and the high affinity receptor (*NK-1*) for its peptides by the same stimulus e.g. cytokine (Table 6). The production of PPT-I peptides could interact with the G-protein coupled NK-1 to activate cAMP pathway (Vaupel, R. *et al.* 1998, *Endocrinology* 123, 2140-2145), consequently regulating *PPT-I* expression

5   through CRE and CRE-like. We first induced *NK-1* with optimal SCF for 36 h (5052±50 molecules/μg total RNA). Since SCF induces endogenous *PPT-I* (Table 6), we used ELISA (Singh, D. *et al, supra*) to quantitate the level of its major translation product, immunoreactive-SP (SP-IR), in stroma cell extracts. The results are presented as the total levels of SP-IR in 1 ml of cell extract, obtained from one confluent stromal

10   layer, grown in 25 cm² tissue culture flask. The results showed 115±8 pg/ml (n=5, ±SD) of SP-IR after 36 h in SCF-stimulated stromal extracts whereas, extracts from unstimulated stroma consisted <1 pg/ml. We therefore asked whether SCF-mediated production of SP could stimulate the cells through autocrine mechanism. To address this, we incubated the SCF-stimulated cells with 10 nM of an NK-1-specific antagonist

15   (CP-99,994) and then quantitated luciferase activity. To ensure that the manipulation by the transfection did not blunt the production of endogenous Substance P, we determined Substance P levels in five different experiments in which stroma was stimulated with SCF. SP-IR at time 0 and 4 h post-transfection were 110±18 and 122±14 pg/ml, ±SD respectively. Despite the high levels of SP-IR, luciferase activity

20   was significantly reduced in the presence of the antagonist (Figure 7A). Antagonist alone did not affect luciferase activity compared to transfectants with vector alone. The results of these experiments show that NK-1 is at least partly required for the activation of *PPT-I* promoter by SCF.

We next determined if SCF could be initiating a response so that PPT-I is able

25   to auto regulate its own expression. To address this, we stimulated bone marrow stroma with 1 nM of the major PPT-I peptide, substance P, SP (Sigma), and then determined the levels of *PPT-I* mRNA by quantitative RT-PCR. Compared to undetectable *PPT-I* mRNA in unstimulated cells, cultures with SP showed significant increase in β-PPT-I, *p*<0.01 (Table 7). NK-1-specific antagonist (CP-99,994) did not

30   completely blunt the effects of SP since at 10 nM, there was only 7-fold reduction (Table 7). The data showed that SP, at least partly through NK-1 could mediate its own expression. Similarly, induction of endogenous *PPT-I* by SCF is bluned by CP-99,994

(Table 7). In summary, the results in this section used two different approaches, transient transfection and induction of endogenous *PPT-I*, to show that SCF could induce *PPT-I* directly and/or indirectly through activation of the G-protein-coupled NK-1 (Figure 7B).

Table 7. Induction of *PPT-I* by substance P (SP)

Stimuli	β-*PPT-I* (molecules/μg total RNA)
Unstimulated or CP-99,994	<1
SP	284 ± 12*
SP + CP-99,994	42 ± 4
SCF	1324 ± 76**
SCF + CP-99,994	119 ± 8

BM stroma were stimulated with 1 nM SP, 8 ng/ml SCF and/or 10 nM CP-99,994 for 16 h. After this, β*PPT-I* levels were quantitated using total RNA. *$p<0.01$ vs. SP + CP-99,994, n = 6, ±SD; **$p<0.01$ vs. SCF + CP-99,994. The results are shown with optimum concentration of CP-99,994, derived from dose-response studies. The levels of *NK-1* mRNA in stroma stimulated with SCF in the presence or absence of CP-99,994 were 49,195 ± 110 and 5,085 ± 140 molecules/μg total RNA respectively.

Example 11.

Cell-specific activity of *PPT-I* promoter

PPT-I is expressed with different efficiency and by different stimuli in particular tissues (Rameshwar, P. 1997, *supra*; Hennig, I., *supra*; Hart, R.P., *supra*). In bone marrow stromal cells, although macrophage and fibroblasts express endogenous PPT-I when stimulated by IL-1α, the levels in macrophage are relatively higher than fibroblasts. Similar differences are observed in IL-1α-stimulated bone marrow and skin fibroblasts. The data described for Figure 7B indicate that *NK-1* expression regulates *PPT-I* expression through signaling of cAMP. Since the expression of *NK-1* is different in bone marrow stroma, inducible, (Ramewshwar, P. 1997, *supra*) and neural cells, constitutive (Rameshwar, P. *et al* 1997, *supra*), we determined if there is tissue and/or cell specificity in its regulation. We chose relevant cells based on the role of

PPT-I in areas of major clinical interests such as breast cancer, hematological disorders and brain-associated injuries and/or dysfunctions. Thus, we used fibroblasts from bone marrow and skin, undifferentiated neuroblastoma (SY5Y) and mammary epithelial cells. Since Intron 1 and Exon 1 could have regulatory regions (Figure 3), we used

5   *PPT-I-p1.2* and Upstream/N0 for cell transfection. Because transfection efficiency could vary depending on the cell source, for comparison purposes, cells were co-transfected with pβ-gal and each transfection normalized with β-gal activity. The results, summarized in Figure 8 indicate that mammary epithelial cells transfected with pGL3-*PPT-I*-p1.2 showed significant increase in luciferase compared to fibroblasts and

10   SY5Y. There was no difference in reporter activity in the two sources of fibroblasts. We also observed comparable luciferase activities in transfected fibroblasts and SY5Y.

      While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be

15   limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.